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Interactions between free-living amoebae and foodborne pathogens

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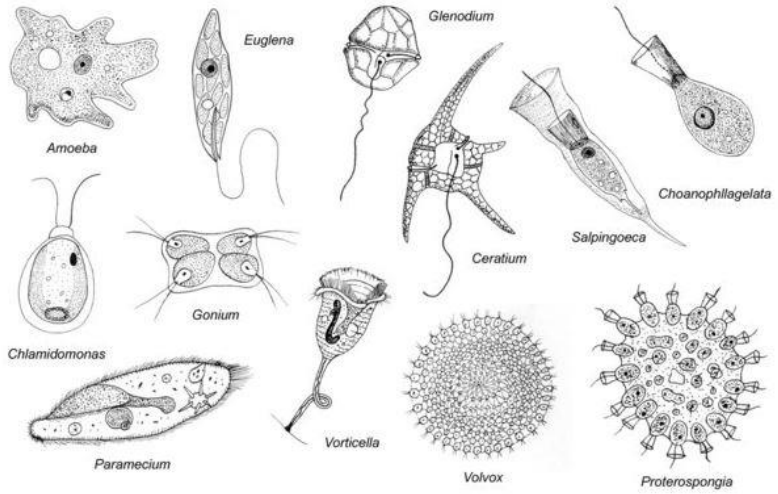
List of abbreviations

AFS	Automatic Freeze Substitution
ARB	Amoeba Resistant Bacteria
ATCC	American Type Culture Collection
CFU	Colony Forming Units
CmeC	*Campylobacter* multidrug efflux pump protein
DOC	Dissolved Organic Carbon
dot/icm	defective in organelle trafficking/ intracellular multiplication
EFSA	European Food Safety Authority
EHEC	Enterohaemorrhagic *E. coli*
EMPact	Electron Microscopy High Pressure Freezing device (Leica)
FeoB	Ferrous iron transport B
FLA	Free-living amoebae
FLP	Free-living protozoa
HPF-AFS	High Pressure Freezing and Automatic Freeze Substitution
HS	High Saline
HUS	Haemolytic Uraemic Syndrome
LCV	*Legionella* Containing Vacuole
LPS	Lipopolysaccharide
MH	Mueller Hinton
MOI	Multiplicity Of Infection
MPN	Mean Probable Number
OmpA	Outer membrane protein A
PAS	Page's Amoeba Saline
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
p.i.	post infection
PW	peptone water
PYG	Proteose peptone Yeast extract Glucose
pYV	*Yersinia* virulence plasmid

rDNA	ribosomal deoxyribonucleic acid
RAS	Rhizaria, Alveolata and Stramenopiles
SPI1/2	*Salmonella* Pathogenicity Island 1/2
STEC	Shiga Toxin producing *E. coli*
stx	shiga toxin
T3SS	Type III Secretion System
TEM	Transmission Electron Microscopy
TSB	Tryptone Soya Broth
VBNC	Viable But Non Culturable bacteria
VTEC	Verocytotoxygenic *E. coli*
YOPI	Young, Old, Pregnant, Immunocompromised

CHAPTER I

General introduction



1. Free-living protozoa: introduction to amoebae

1.1 Free-living protozoa: definition and characteristics

Free-living protozoa [*proto:* first + *zoa*: animals] are unicellular, eukaryotic, heterotrophic protists[1] (Adl et al., 2005). These ubiquitous microscopic individuals range in size from 2 µm to 25 mm, though some multinucleate, giant protozoa reaching up to 20 cm have been discovered (Hausmann et al., 2003; Khan, 2009). They typically feed on bacteria, fungi, algae, other protozoa or dissolved organic particles (Sigee, 2005). Nutrient uptake occurs by phagocytosis - the engulfment of a solid particle by a receptor dependent process - or pinocytosis, the non-specific uptake of fluid and dissolved compounds.

Traditionally, free-living protozoa have been classified in three morphogroups: flagellates, ciliates and amoebae (Figure 1). They are primarily distinguished by their motility but also differ in internal fine structure, cell size and feeding mode (Sigee, 2005).

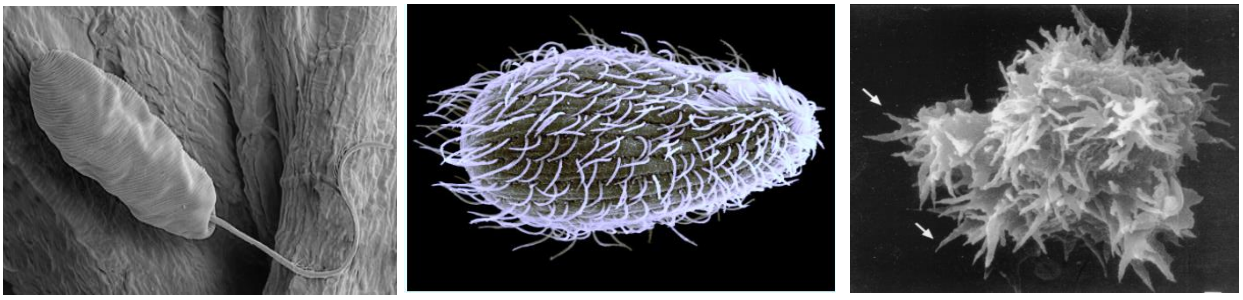


Figure 1. Scanning electron microscopy image of a flagellate, ciliate and amoeba
From left to right: *Euglena*, *Tetrahymena thermophila*, *Acanthamoeba castellanii*
Figures taken from https://yooniqimages.com/images/detail/207356013/Creative/euglena-flagellate-protozoan-sem, http://faculty.jsd.claremont.edu/ewiley/about.php, Marciano-Cabral et al 2003

Flagellates are rather small protozoa (5-20 µm) containing one to eight whip-like structures. These so-called flagella (s. flagellum) are membrane bound filamentous projections which are composed of series of parallel microtubuli, exhibiting a 9+2 arrangement. In addition to locomotion, flagella can also be used for predation[2], filter feeding[3] and attachment to the substrate (Sigee, 2005). Examples of flagellates include *Cercomonas, Chilomonas* and the sessile *Actinomonas*.

[1] eukaryotes with a unicellular level of organization, without cell differentiation into tissues (Adl et al 2005)
[2] the active pursuit and ingestion of motile preys
[3] generation of filter currents

Ciliates are usually between 15-200 µm and are covered with rows of numerous cilia (thread like structures) which are used for locomotion and feeding. The cilia are arranged in longitudinal, oblique or spiral rows and the beats of these cilia are coordinated and function like oars to propel the organism. Cilia and flagella are made up of the same protein components and are actually equivalent structures. Cilia however are shorter (ca. 0.25 µm vs 100-200 µm) and tend to occur in larger numbers (Sigee, 2005). Ciliates such as *Paramecium*, *Colpoda* and *Tetrahymena* are frequently isolated from the environment.

Amoebae are generally smaller than 200 µm and mostly lack cilia or flagella. They typically crawl along a solid substratum by projection of cytoplasmic extensions i.e. pseudopodia. Pseudopodia are also used for predatory food capture and ingestion. Some well-known examples of amoebae are *Acanthamoeba, Hartmannella* and *Vannella*. However, not all amoebae are motile, active predators. The floating, star-like *Heliozoa* have stiffened pseudopodia, which are only used for passive diffusion, and not for active motility. Several amoebae produce an external shell, referred to as test or lorica, which consist of organic and anorganic compounds secreted by the amoeba or trapped from the environment (Sigee, 2005). Moreover, certain amoebae such as *Hyperamoebae* have a flagellated live stage and are called amoeboflagellates.

The focus of this thesis is on **free-living amoebae (FLA)**, which do not have an obligate parasitic life cycle stage. However, certain FLA species such as *A*. *castellanii*, *Balamuthia mandrillaris* and *Naegleria fowleri* can occasionally cause infections in humans. Moreover, FLA frequently harbor (pathogenic) intracellular bacteria (cf. 2.2)

1.2 Diversity and classification of protozoa and FLA

Classification of protozoa, has been and still is an issue of debate. The **term protozoa** was first introduced by Goldfuss in 1818 to describe a class of primitive invertebrates in the established kingdom of Animalia and has been in use and modified ever since. It became clear that protozoa are a paraphyletic group that neither belong to animals, plants, nor fungi. Over time, scientists argued that protozoa should be considered as a phylum, subkingdom or even a kingdom of their own (historical overview: Scamardella, 1999).

In 2005, Adl and colleagues proposed a complete new classification for eukaryotes based on biochemical, morphological and molecular data. This classification abandoned the traditional Linnean category names of Kingdom, Phylum, Class and Order (Adl et al., 2005) and has been modified several times (Adl et al., 2007; Burki et al., 2007; Adl et al., 2012; Burki et al., 2012). The new classification scheme, contains five supergroups: Opisthokonta, Amoebozoa, Excavata, Archaeplastida, and RAS (Rhizaria, Alveolata, Stramenopiles) (Figure 2). Protozoa are dispersed over all but one - Archaeplastida - supergroups. The morphogroups - ciliates, flagellates and amoeba - do not always correlate with specific phylogenetic entities. Ciliates form a monophyletic group within the Alveolata (Adl et al., 2005), whereas flagellates are known to be highly polyphyletic (descended from more than one ancestor, (Sleigh, 1989). **Amoebae** are mainly found in the Amoebozoa and Rhizaria supergroups (Adl et al., 2005). However, phylogenetic and metagenomics data demonstrated that not all eukaryotic organisms can be placed in the 5 supergroups. These unclassified lineages represent a large variety of organism including free-swimming or gliding biflagellated cells and amoeboflagellates (Pawlowski, 2013).

The high diversity, the disagreements over the species concept and the paraphyletic or polyphyletic nature of many groups makes protozoa classification challenging. As the number of habitats being sampled and the amount of molecular data generated will increase, the classification of protozoa will undoubtedly be revised in the future.

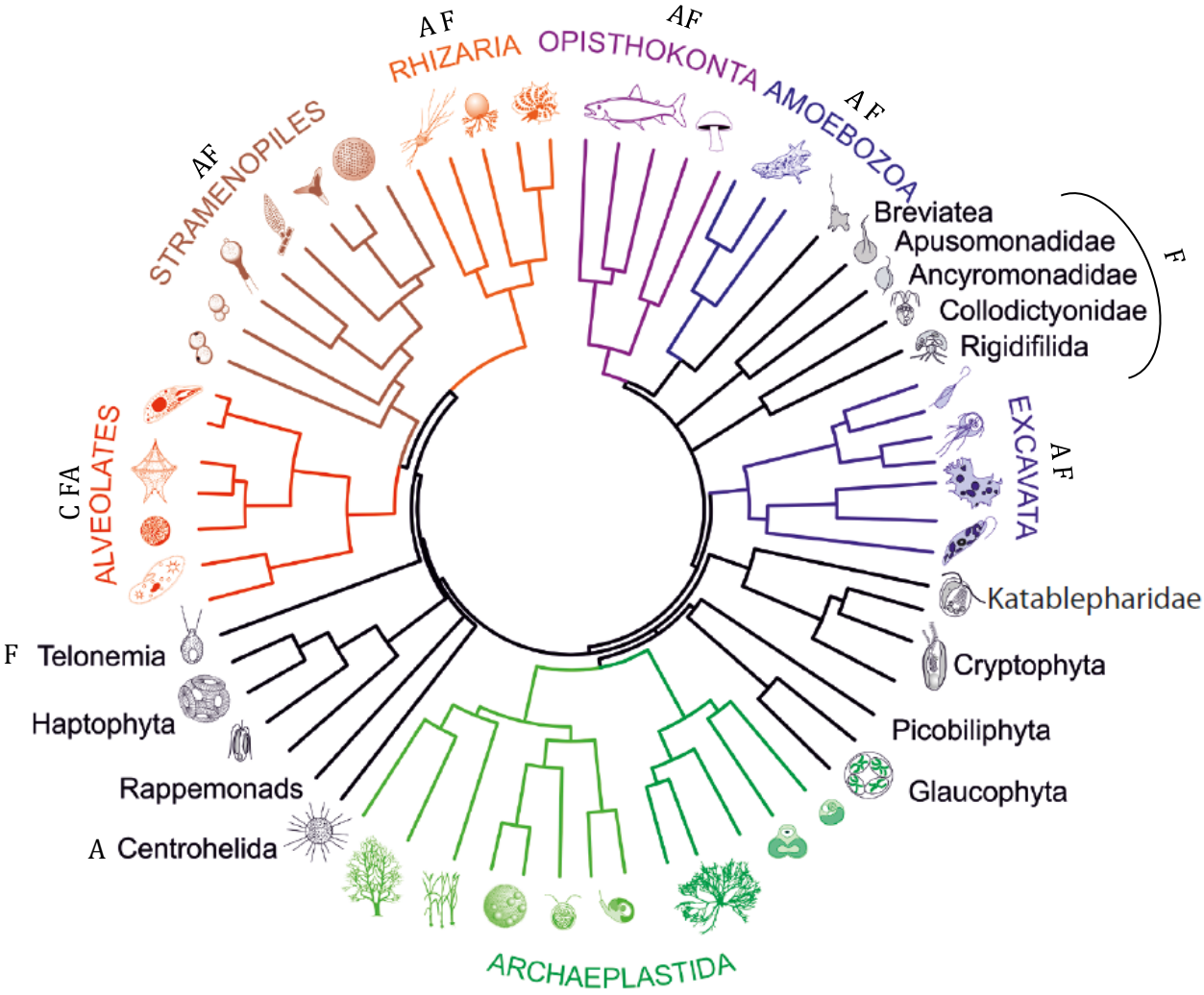


Figure 2. Classification of eukaryotes into supergroups and small lineages that branch outside these groups
Protozoan morphogroups are indicated; C: ciliates, F: flagellates, A: amoebae
Figure adapted from (Pawlowski, 2013)

1.3 Occurrence of free-living amoebae

1.3.1 General

Free-living amoebae are ubiquitously present in natural aquatic and soil ecosystems (Hausmann, 2003). Their occurrence and diversity depends on biotic (e.g. predator-prey, competition, parasitism) and abiotic factors (e.g. salinity, pH, temperature, humidity, light, concentration of ions/dissolved gasses) (Khan, 2009). Many amoebae survive unfavorable environmental conditions including desiccation, biocides and temperature and pH fluctuations by transforming into a resting stage, i.e. a cyst. By transformation into cysts, amoebae can become airborne and transmitted to new habitats (Rogerson and Detwiler, 1999). In addition, FLA can sometimes cause infections themselves, but are also important from a public health perspective as they can harbor pathogenic endosymbionts and often resist disinfection treatments (cf. 2.2).

1.3.2 Natural environments

Free-living amoebae have been isolated from diverse natural habitats including air (Rivera et al., 1991; Rodriguez-Zaragoza et al., 1993), soil (Bass and Bischoff, 2001; Nacapunchai et al., 2001), sea sediments (Hamels, 2003; Pede, 2012), marine waters (Arias Fernandez et al., 1989) and fresh water (Mahmoudi et al., 2015; Armand et al., 2016). Moreover, they were observed in extreme environments such as geothermal springs (Aguilera et al., 2010), natural hot springs (Lekkla et al., 2005), caves (Mulec et al., 2012) and arid and salty soils (Robinson et al., 2002). Many protozoa observed in these extreme environments are in the cyst stage, but are still viable.

Protozoa, among which amoebae are widely recognized as predators of bacteria (Barker and Brown, 1994), controlling bacterial populations in a wide variety of soil and water habitats. They show marked prey preferences and therefore determine the amount and variety of microbial species (Hamels, 2003; Huws et al., 2005; Dopheide et al., 2011). Feeding rates can go up to 1465 bacteria/amoeba/h, 300 bacteria/flagellate/h and 1000 bacteria/ciliate/h (Sigee, 2005; Parry et al., 2007).

In **aquatic environments**, amoeba are involved in the aquatic microbial loop, where they form trophic bridges that transfer the nutrients (carbon and other nutrients) and energy of bacteria and algae further up in the food web. They ingest bacteria which have taken up dissolved organic carbon (DOC) derived from decomposition of plants and other organisms. In turn, protozoa are ingested by other protozoa, zooplankton and invertebrates, transferring energy to higher trophic levels (Finlay and Esteban, 1998). Moreover, breakdown of bacteria by protozoa results in production of new DOC and excretion of nutrients, nitrate, phosphate and ammonium (Sigee, 2005), which may stimulate the growth of bacteria and algae again.

Soil ecosystems are inhabited by protozoa that are adapted to desiccation and mechanical stresses e.g. testate amoeba or cyst forming FLA. Amoebae are key organisms for soil fertility, as they make nitrogen available to algae, plants and other soil organisms (Bonkowski, 2004). The bacteria they eat contain more nitrogen than the protozoa can use, so they excrete the excess as ammonium. Soil inhabited by *Acanthamoeba* and bacteria showed significantly greater mineralization of carbon, nitrogen, and phosphor compared to soil containing only bacteria (Ronn et al., 2002).

Though it has been shown that FLA play an essential role in natural ecosystems, in the past they have often been neglected in standard microbiome studies, which are mainly based on 16S sequencing, and in *in vitro* community experiments.

1.3.3 Anthropogenic environments

1.3.3.1. Human related water systems

Free-living amoebae have been detected in various anthropogenic environments including hospital water networks (Rohr et al., 1998; Trabelsi et al., 2015), dental unit water systems (Hikal et al., 2015), industrial textile wastewater (Ramirez et al., 2014), cooling towers (Behets et al., 2007; Declerck et al., 2007), swimming pools (De Jonckheere, 1979; Rivera et al., 1993; Vesaluoma et al., 1995), cars (Simmons et al., 1999), condense water of a space station (Ott et al., 2004) and household waters (Stockman et al., 2011).

Free-living amoebae play a key role in biological water treatments plants, where they control bacterial numbers in the activated sludge, remove excess organic matter and contribute to the flocculation process (Witthauer, 1980; Hawkes, 2013).

1.3.3.2. Food related environments

Amoebae form part of the '**in-house microbiota'** of food and food related environments (Vaerewijck et al., 2014). Baré and colleagues observed different taxa, belonging to all functional groups (flagellates, ciliates, amoeba) in **commercial broiler houses**. Diversity and composition of the protozoan communities was related to habitat type, with water supply samples having a higher diversity and containing more amoebae than dry samples (e.g. litter) (Baré et al., 2009; Baré et al., 2011). **Meat cutting plants** have also been shown to contain a high diversity in FLA. Vaerewijck and colleagues encountered flagellates, ciliates and amoebae on various surfaces in meat cutting plants. Sampling sites rich in inorganic material or with high moisture content were more likely to be FLA-positive (Vaerewijck et al., 2008). Currently, free-living protozoa are not monitored during standard food safety monitoring procedures, so it remains unknown if and how protozoa influence the bacterial communities (incl. spoilage and pathogenic bacteria) in food processing environments.

At the consumer level, **refrigerators** are inhabited by FLA, with surfaces of the vegetable trays and discharge gutters being the most frequently occupied by free-living protozoa. Overall, flagellates and amoebae were the dominant groups in the domestic refrigerators, while ciliates were mainly located on the vegetable trays. The FLP-positive status of refrigerators could be linked to a high bacterial load (Vaerewijck et al., 2010).
Dishcloths are also occupied by diverse protozoan communities. Of all examined dishcloths, 89% were positive for FLP, with flagellates being the most abundant group, followed by amoebae and ciliates respectively (Chavatte et al., 2014).

1.3.3.3 Food and drinking water

Data on occurrence of FLA on food is scarce and is mainly focused on **vegetables**. FLA have been detected on lettuce, carrots, cauliflower, radishes, scallions, spinach, and tomatoes (Rude et al., 1984; Sharma et al., 2004; Gourabathini et al., 2008; Vaerewijck et al., 2011). The number of protozoa on leafy vegetables was high, with flagellates being more abundant than amoebae and ciliates (Vaerewijck et al., 2011). A recent study revealed a high diversity on various vegetable seed sprouts (e.g. alfalfa, cress, beetroot), whereby community composition and abundance differences could be linked with specific seed sprout types (Chavatte et al., 2016b). Seasonality and company had less influence on the protozoa community composition.

Various studies report the presence of FLA in tap **water** (Hoffmann and Michel, 2001; Thomas et al., 2006; Bagheri et al., 2010) and bottled mineral water (Maschio et al., 2015). Currently, there is no data available about the presence of FLP on/in meat and in dairy.

1.3.4 Humans and animals

With the exception of amoebal keratitis registrations (cf. 1.4.2), the occurrence and diversity of FLA on/in humans has been rarely studied. More than 80 % of healthy human individuals have been tested seropositive for antibodies of *Acanthamoeba* in their blood (Chappell et al., 2001), demonstrating that exposure to FLA is common. Free-living amoebae have been detected in stool (Jadin et al., 1973; Zaman et al., 1999; Bradbury) and nasal samples of healthy humans (Corsaro and Venditti, 2015). *Vermamoeba* and *Acanthamoeba* were among the most encountered amoebae in human samples.

Some protozoa are part of the normal microbial gut flora of animals and insects (Tokuda et al., 2014). Feces of snakes, fish, pigeons, ducks, squirrels, gulls, musk rats, toads, frogs and pigs were all positive for FLA (Jadin et al., 1973; Lorenzo-Morales et al., 2007; Niyyati et al., 2009; Chavatte et al., 2016a). Though the function of FLA in the gut of these animals has not been unraveled yet, it can be hypothesized that their grazing behavior helps to shape and regulate prokaryotic populations.

1.4 *Acanthamoeba*

Free-living amoeba and especially *Acanthamoeba castellanii* are ubiquitous in natural and anthropogenic environments where they encounter bacteria, viruses, algae and fungi amongst others, and interact with other conspecifics and other FLP. It is a highly relevant genus for testing interactions with foodborne pathogens, since both microorganisms share the same habitat. *Acanthamoebae* are considered as a potential reservoir, vector and virulence training ground for intracellular, digestion resistant bacteria (cf. 2.4.).

1.4.1 Morphology

The free-living amoeba *Acanthamoeba* was discovered in 1930 by Castellani. The prefix, 'acanth' (greek 'acanth' means spike) refers to the characteristic spine like pseudopodia, also called acanthapodia (Figure 3A). These cytoplasmatic projections are involved in adhesion, motility and capturing prey (Khan, 2009).

Acanthamoeba is characterized by two life stages: a vegetative trophozoit (Figure 3A) and a dormant cyst stage (Figure 3B). The single nucleated trophozoites are amorphous and have a mean diameter of about 25 µm (Khan, 2009). Their morphology can change according to culture conditions and they are generally larger in size when cultured with bacterial prey. Feeding occurs through phagocytosis and pinocytosis. These amoebae move relatively fast on solid surfaces (0.8 µm/sec). Under optimal growth conditions, *i.e.* neutral pH, 30°C and an osmolarity between 50-80 mOsmol (Khan, 2009), *Acanthamoeba* divides by binary fission. Its generation time can vary between 8-24 h. Thermotolerant strains of *Acanthamoeba* have been described. These have an optimal growth temperature of 37°C, and cysts can resist up to 7d at 45°C and up to 10 min at 80°C (Storey et al., 2004).

It has been estimated that 25% of *Acanthamoeba* in nature harbor intracellular bacteria ((Winiecka-Krusnell and Linder, 2001). Upon binary fission, those bacteria are passed to the progeny (Khan, 2009).

In unfavorable environmental conditions, trophozoites become encapsulated in a robust **cyst,** a process called encystment. Encystment involves many structural and functional changes and is characterized by dehydration, secretion of carbohydrates, proteins and

soluble aminoacids. During encystation the amoeba produces a double-layered cyst wall, which comprises an external wall (ectocyst) and an inner wall (endocyst). Both layers are composed of fibrous material such as cellulose, xylofuranose, xylopyranose, glucopyranose, mannopyranose and galactopyranose (Aqeel et al., 2013). On the surface of the cyst wall, several pores, ostioles, can be observed, through which the amoeba remains in contact with the outer environment. When favorable conditions return, cysts revert back to the active trophozoit form (excystment), leaving the empty cyst walls behind. Intracellular, digestion resistant bacteria like *Legionella pneumophila* and *Mycobacterium spp*. can survive the encystment process. Whereas *Legionella* are located inside the cysts, *Mycobacterium* can be found in between the two cyst walls (Steinert et al., 1998; Price et al., 2014). As the majority of the studies with *Acanthamoeba* cysts are performed *in vitro*, information on factors which stimulate en-and excystment *in situ* is scarce.

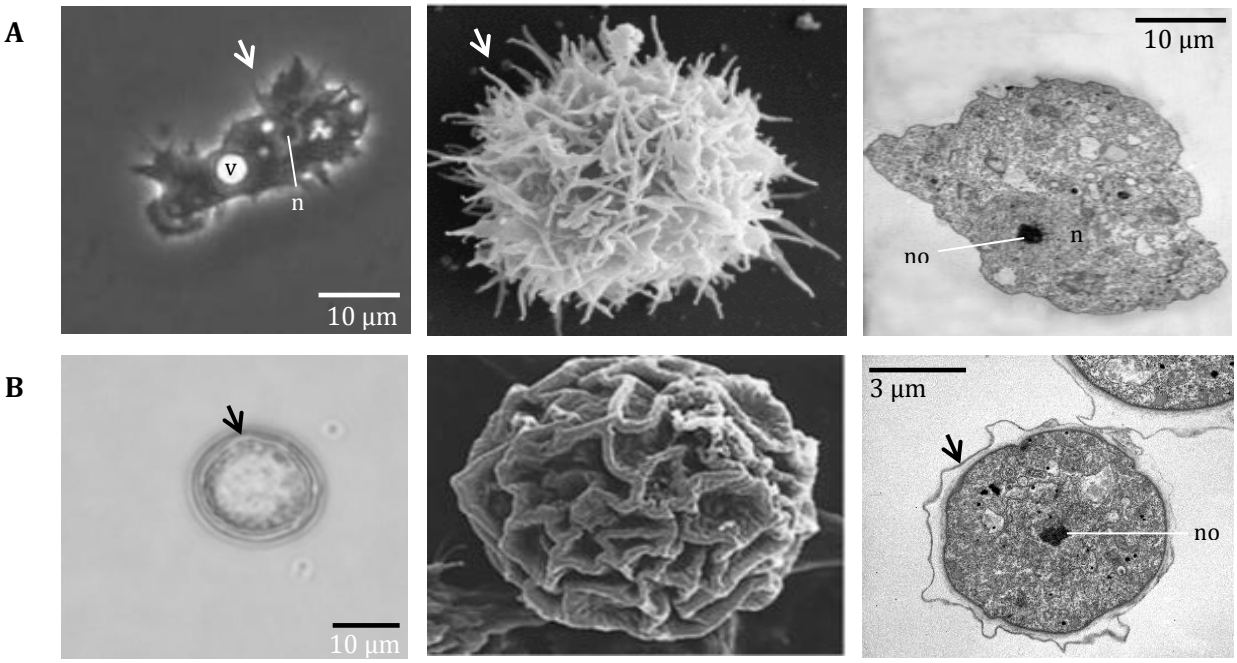


Figure 3. *Acanthamoeba castellanii* trophozoit (A) and cyst (B)
From left to right: light microscopy, scanning electron microscopy, transmission electron microscopy.
White arrow: pseudopodia, black arrow: double layered cyst wall, n: nucleus, no: nucleolus, v: vacuole
Own pictures, Scanning electron microscopy pictures taken from Khan 2009

1.4.2 Pathogenicity

Despite its ubiquitous nature, *Acanthamoeba* is considered as a biosafety level II pathogen. *Acanthamoeba* is the causative agent of granulomatous amoebic encephalitis, a rare but fatal central nervous system disease, and amoebic keratitis, a sight-threatening infection of the cornea (Marciano-Cabral et al., 2003; Lorenzo-Morales et al., 2015). However, not all amoebae belonging to the genus *Acanthamoeba* are pathogenic. The genus is currently classified into 20 different genotypes (T1-T20) based on 18S rRNA sequencing (Corsaro et al., 2015) and the majority of the strains associated with keratitis and granulomatous encephalitis belong to the T4 genotype. Although the trophozoites are responsible for the infections, the cysts ensure the persistence of the pathogen in the environment (Chavez-Munguia et al., 2005).

Wearing contact lenses is the leading risk factor for amoebic **keratitis** (incidence: 0.01-1.49/10000, (Khan, 2009)) even among immunocompetent persons. Factors that promote the infection are poor contact lens hygiene, corneal abrasions or home-made saline solutions. Amoebic keratitis is difficult to diagnose and to treat because amoebic cysts are resistant to many drugs. **Granulomatous encephalitis** is a rare disease, with over 150 described cases worldwide (Schuster and Visvesvara, 2004) and primarily affects people with compromised immune systems. By inhalation through the nose or lungs or entry through skin lesions, *Acanthamoeba* enters the human body, crosses the blood-brain-barrier and is spread to the central nervous system where it causes e.g. fatal oedema and necrosis. The pathogenicity and host responses are not completely unraveled and reports of successful treatments of this infections are few.

1.4.3 Model eukaryote

Acanthamoeba has long been studied as a single-cell model eukaryote for population dynamics and cell biological processes such as the actin cytoskeleton-based motility (Pollard et al., 1970; Pollard and Korn, 1973) and phagocytosis (Allen and Dawidowicz, 1990; Alsam et al., 2005). These FLA share similarities in cellular organization with mammalian models, are easy to study, are cultivable to high numbers, are genetically well-defined, are resistant to various environmental conditions, and are generally cheaper to maintain and have fewer

ethical implications compared to mammalian cell lines and mammals (Montagnes et al., 2012).

By using amoebal coculture and amoebal enrichment of environmental samples, several new intracellular bacterial species and viruses have been discovered (Jacquier et al., 2013; Tosetti et al., 2014). Amoebae possess recognition, uptake and digestion mechanisms which may be comparable to those found in macrophages. Therefore, this amoeba might be used to study virulence properties of associated microorganisms (Tezcan-Merdol et al., 2004; Bleasdale et al., 2009; Van Waeyenberghe et al., 2013). *Acanthamoeba* can be artificially induced to enter or exit the dormancy stage (cysts), a feature which could be applicable to human cancer research (Baig et al., 2015).

2. Free-living amoebae: bacterivory and grazing resistance

The outcome of the FLA-bacteria interaction is complex and dynamic and depends e.g. on environmental conditions, bacterial virulence, FLA species/strain identity and the density and ratio of amoeba:bacteria. Interactions may initially lead to destruction of either the bacteria or the amoeba, temporary retention of resistant bacteria or establishment of a true endosymbiotic relationship.

2.1 Amoebal bacterivory

Free living amoebae are generally regarded as **predators** of bacteria. Feeding on bacteria occurs through phagocytosis during the trophozoit stage. They preferentially feed on Gram negative bacteria, however, in the absence of any choice, they take up any available bacteria (Khan et al., 2014). The underlying cellular and molecular mechanisms of selective feeding are not know yet. Factors likely involved in this process are (i) pheromones/terpenes produced by certain bacteria or by-products of bacterial metabolism that attract or deter protozoa; (ii) specific, functional chemoreceptors on the FLA membrane to locate food; (iii) size and morphological features of the prey; (iv) biochemical properties of bacterial surface such as lipopolysaccharide and peptidoglycans; (v) presence/absence of lytic enzymes required to degrade a particular prey (Khan et al., 2014). Moreover, it was shown that related amoebae (99% similarity in 18S rDNA loci) can have different feeding preferences when they are found in different environments (Wildschutte and Lawrence, 2007), whereas phylogenetic unrelated amoeba can show similar feeding preferences. These findings suggest that the environment in which the amoeba reside plays a pivotal role in shaping its feeding preferences.

In addition to food particles, FLA can also take up non-nutritive particles like latex beads. However, these latex beats will follow a different intra-amoebal pathway than bacteria, indicating that FLA can discriminate between digestible and indigestible particles. Particles greater than 1 µm are captured by pseudopodia and engulfed as such. Smaller particles are accumulated at the surface until they reach a critical aggregate size, after which they are ingested collectively (Figure 4). The membrane enclosed food particles bud-off in the

cytoplasm and form the phagosome. Phagosomes containing non-digested latex beads are exocytosed (Khan, 2009), whereas phagosomes containing bacteria will fuse with lysosomes containing hydrolytic enzymes which helps to digest the bacteria. Although amoebal ingestion rates can be quite high i.e. 400 bacteria/amoeba/h, they rarely eradicate a complete prey community. Mechanisms such as physical refuge (e.g. small soil pores), switching to another prey (attack depends on prey density) and increased replication of a prey will compensate the killing (Alexander, 1981).

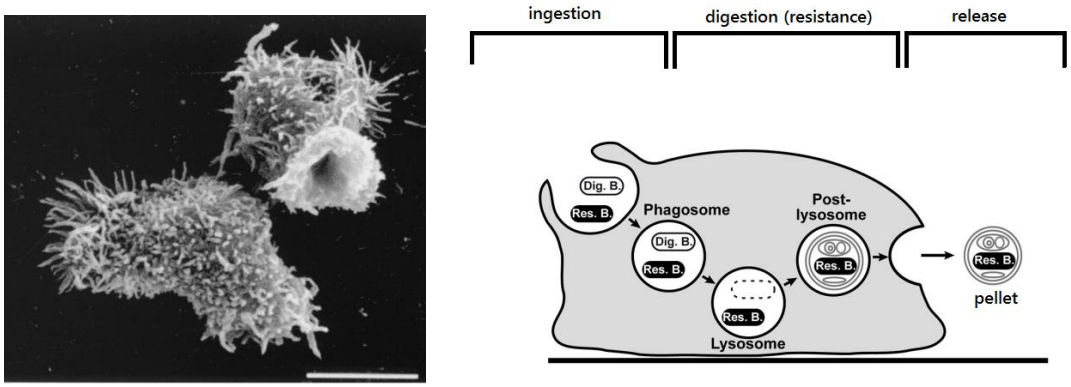


Figure 4. Phagocytosis by *Acanthamoeba*
Left: pseudopodia forming a food cup. Picture taken from Marciano-Cabral et al 2003.
Right: Digestion pathway inside *Acanthamoeba.* Dig. B: digested bacteria, Res.B: resistant bacteria
Figure taken from (Denoncourt et al., 2014).

2.2. Amoeba resistant bacteria

Co-evolution and selection led to the evolution of strategies to prevent amoebal uptake and/or digestion in bacteria (Declerck and Ollevier, 2007). It has been shown that these **amoeba resistant bacteria (ARB)** can be detected within days in response to selective protozoan grazing (Matz and Kjelleberg, 2005). However, the former authors did not investigate the underlying mechanism for the rise of ARB. A possible explanation is that spontaneous mutations in a clonal bacterial population led to fitter ARB which became abundant upon amoebal grazing. Alternatively, resistance could be due to induction of transcriptional activation of unknown genes that were repressed in the initial population. Finally, it's also possible that the initial population was non-clonal and that some resistant bacteria were already present in low concentrations.

Two divergent adaptive strategies of the bacteria can emerge: pre-ingestional adaptations and post- ingestional adaptations.

Pre-ingestional adaptations include alteration in cell size and morphology (e.g. formation of bulky filamentous cells (Pernthaler et al., 1997; Gaze et al., 2003), increased motility (Matz and Jurgens, 2005), modification of surface molecules (Wildschutte et al., 2004), formation of microcolonies and secretion of toxic compounds (Matz et al., 2004). These strategies prevent bacterial uptake by protozoa.

In natural and anthropogenic environments, bacteria are commonly found as adherent communities embedded in an extracellular polymeric matric, known as **biofilms** (Huws et al., 2005). Protozoan grazing is a major factor controlling biofilm community composition (Huws et al., 2005). Some biofilm associated bacteria protect themselves by the production of toxins. For instance, it has been demonstrated that bacteria inside marine biofilms can produce violacein, an alkaloid toxin, which inhibits protozoan grazing by inducing cell death (Matz et al., 2008).

Post-ingestional adaptations include preventing fusion between phagosome and lysosome, escape out of the phagosome into the cytoplasm or digestion resistance (e.g. resistance to low pH). Bacteria that resist digestion can establish an intracellular association with amoeba, which can be either transient (facultative intracellular bacteria) or stable (obligate intracellular bacteria). These associations can be neutral, mutualistic[4], commensalistic[5] or parasitic[6].

At a certain moment, internalized, non-digested bacteria are released (or trigger their **release)** into the environment. They can be released as cells or as 'amoebal packages', i.e. aggregates in membrane-bound vesicles, which are also called fecal pellets. Amoebae are known to produce fecal pellets or vesicles as part of their normal digestive process (Paquet et al., 2013; Denoncourt et al., 2014). These fecal pellets contain undigested compounds and organic nutrients, and their exact composition, size, and morphology, depends on the protozoa and the trophic conditions (Denoncourt et al., 2014).

[4] both organisms benefit from each other
[5] one organism benefits from the other without affecting it
[6] one organisms benefits while the other is harmed

Bacteria that resist digestion can also be packed in these structures and are expelled by exocytosis or cell lysis (Abd et al., 2003; Brandl et al., 2005; Gourabathini et al., 2008; Baré, unpublished). Amoebal packages containing bacteria range in size from 2 to 6 µm (Berk et al., 1998) and are formed of several layers of lipid membranes (Figure 5 , Denoncourt et al 2014). The lipids constituting the secreted packages are of amoebal origin (Paquet et al., 2013).

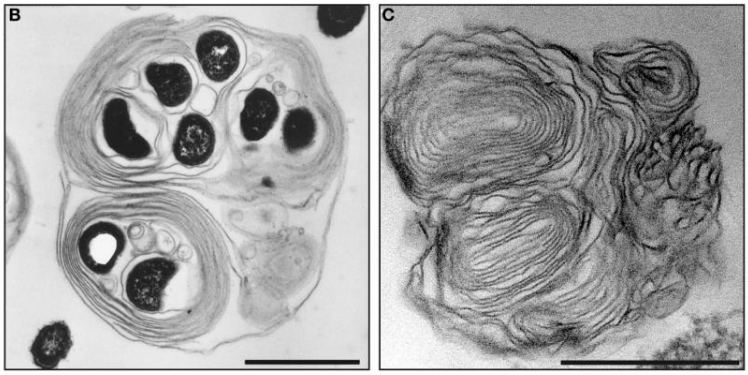


Figure 5 Bacteria packed in amoebal pellets
Left: bacteria (black) inside a multilayered pellet secreted by *A. castellanii*. Right: pellet without bacteria, secreted by *D. discoideum* grown on digestible bacteria. scale bar = 1 µm
Figures taken from Denoncourt et al 2014.

2.3 Endosymbionts

Free-living amoeba and bacteria interact with eachother and co-evolve. Several ARB can establish an enduring, stable intracellular, *i.e*. endosymbiontic relationship with FLA (Figure 6). Endosymbiotic interactions have been described between FLA and members of the Alphaproteobacteria, Betaproteobacteria, Bacteroidetes and Chlamydiae (Horn et al., 1999; Birtles et al., 2000; Horn et al., 2001). These bacteria are able to multiply within the amoebal host. Endosymbionts can be found in the cytoplasm (free or vacuole enclosed), endoplasmatic reticulum or the nuclear envelope. Attempts to culture these bacteria without amoebae and *vice versa* failed. Approximately 26% of the *Acanthamoeba* isolates from human corneal samples and 24% of environmental samples contained (obligate) bacterial endosymbionts (Fritsche et al., 1993).

Evolutionary studies revealed that uptake of a bacterium by a phagotrophic eukaryote and development of a subsequent endosymbiotic interaction, was at the origin of mitochondria and plastids (Rodriguez-Ezpeleta and Philippe, 2006; Martin, 2010). Several transitions are required to transform an endosymbiont into an organel: (i) the phagosomal membrane surrounding the endosymbiont is lost, (ii) the host and symbiont cell division become synchronized, (iii) the endosymbiont genome is reduced either by gene loss or by gene transfer to the host cell nucleus and (iv) a protein import system is developed to translocate proteins from host to endosymbiont (Figure 6).

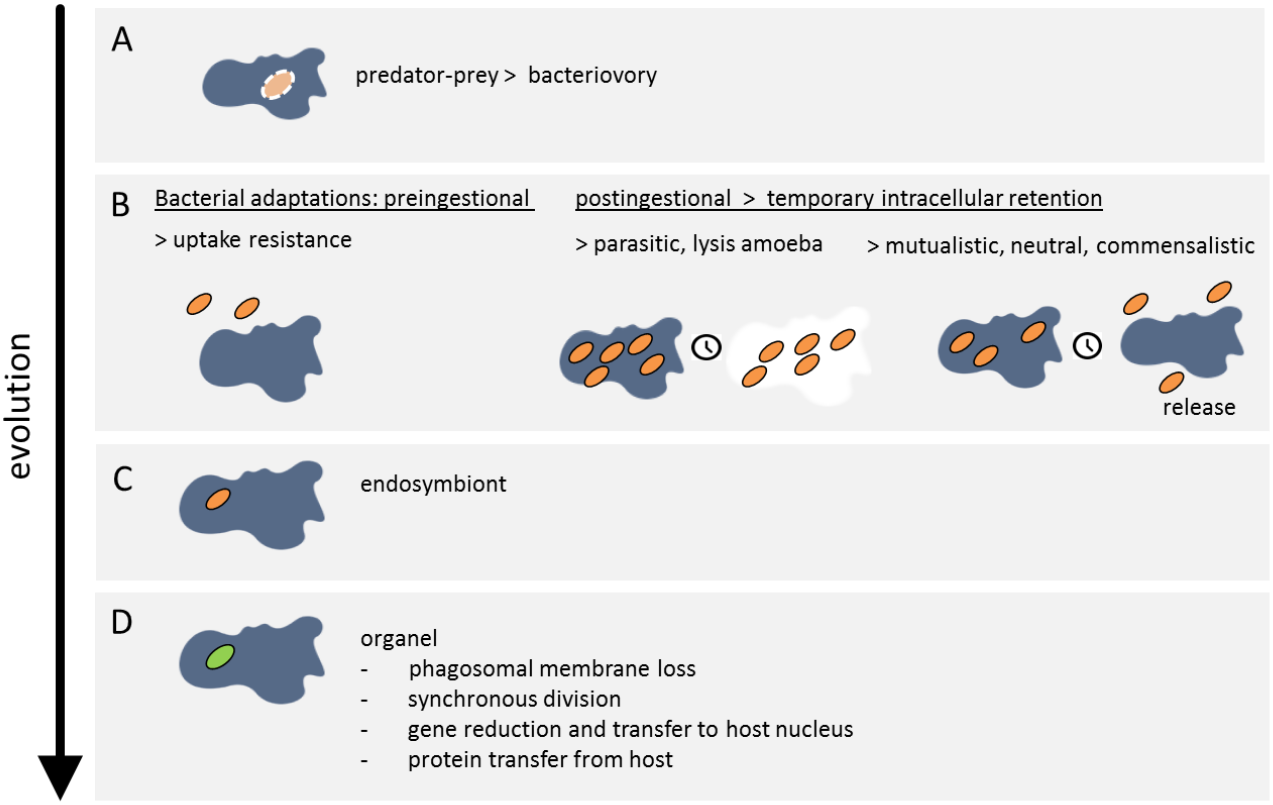


Figure 6. Main evolutionary steps for the transformation of a bacterial prey into an endosymbiont and organel.

2.4 Advantages for amoeba resistant bacteria (ARB)

The development and selection of antipredator mechanisms enables bacteria not only to survive in intracellular and/or extracellular association with protozoa, but also benefit from this association.

An **extracellular** association with amoebae can be beneficial for bacteria as extracellular metabolites and compounds released by amoebae can promote bacterial growth. This has been illustrated by Laskowski. In his study, a phagocytosis-resistant *Vibrio* was shown to grow when cocultured with *Acanthamoeba*, while no viable bacteria could be detected in the cultivation medium alone. The *Vibrio* strain was not taken up by the amoeba, indicating that the growth promotion is mediated by secreted amoebal factors (Laskowski-Arce and Orth, 2008).

An increasing number of *in vitro* studies report that several bacteria can survive and even grow inside amoeba (Greub and Raoult, 2004; Anacarso et al., 2011; Mella et al., 2016), turning them into a potential **reservoir**. Isolation of pathogenic bacteria - including *Bacillus cereus*, *Yersinia pseudotuberculosis* and *Salmonella* spp. - directly from FLA from natural lake and soil samples confirms that amoeba can act as a reservoir (Pagnier et al., 2008) (Evstigneeva et al., 2009). A single amoebal host can even harbor different bacterial species (Michel et al., 2006; Heinz et al., 2007). Free- living protozoa, including *Acanthamoeba,* may be mediators of resuscitation from the Viable But Non Culturable bacteria (VBNC) (Steinert et al., 1997; Garcia et al., 2007). In contrast, intraprotozoal bacteria may also loose there cultivability, but remain viable and pathogenic (Storey et al., 2004; Rahman et al., 2008).

Protozoa can also act as a **vector**, spreading internalized or adhering bacteria to new environments. Amoeba-mediated migration of *Salmonella* on agar plates has been demonstrated by Gaze et al 2003. Whereas *Salmonella* are normally unable to move on the agar surface, they were transported several millimeters per day when cocultured with *Acanthamoeba*.

Free-living amoeba can also facilitate bacterial establishment in hosts, as bacteria inside eukaryotic protozoa (**Trojan Horse)** remain hidden for bacterivores and don’t trigger the immune system immediately.

Brieland and coworkers demonstrated that intra-amoebal *Legionella* can infect mice and cause pulmonary disease (Brieland et al., 1996). In addition, Snelling et al assessed that upon oral administration, intra-amoebal *Campylobacter* could colonize chickens (Snelling et al., 2008).

Bacteria inside trophozoites, inside expelled vesicles and to a greater extent inside cysts are **protected** against deleterious environmental conditions that would normally kill them. These conditions include e.g. heating, antibiotics, disinfectants, and chlorination (Khunkitti et al., 1998; Thomas et al., 2004; Coulon et al., 2010; Dupuy et al., 2014). Protection can be due to lower uptake of the toxic compounds through the protozoan cell membrane, metabolic inactivation of the effective compounds in amoeba, or selection of a resistant bacterial phenotype over time. Even after leaving the protozoan host, bacteria can be more resistant to disinfectants/antibiotics than monoculture bacteria (Barker et al., 1992; Miltner and Bermudez, 2000).

The ability of bacterial pathogens to infect human macrophages is thought to be a consequence of prior adaptation to intracellular growth with various primitive eukaryotic hosts such as FLP. The intracellular conditions of amoebae and macrophages are quite similar and mechanism for uptake and digestion are often comparable ((Van Waeyenberghe et al., 2012; Van Waeyenberghe et al., 2013), cf. 2.5.2). As such, environmental protozoa are considered as **training grounds** for bacteria. In addition, studies revealed that amoeba can serve as a **gene melting** pot where amoeba resistant organisms exchange genes. These horizontal gene transfers may occur between two intracellular bacteria as was demonstrated by McCuddin et al., 2006. In their study a plasmid containing a ceftriaxone resistance gene was transferred from *Klebsiella* to *Salmonella* in rumen protozoa. Moreover, Oguri (2011) reported an increased Extended-Spectrum β-Lactamase gene transfer between two *E. coli* strains in the presence of *Tetrahymena.* Gene transfer has also been described between intra-amoebal bacteria and viruses (Moreira and Brochier-Armanet, 2008) and amoeba themselves can also transmit genes to viruses and intracellular bacteria and vice versa. The latter was revealed by Moliner (2009), who performed phylogenetic analyses on the obligate intra-amoebal bacterium *Legionella drancourtii* and concluded that its malate synthase gene was transmitted to the amoebae *Dictyostelium discoideum*.

Currently there are no studies reporting gene transfers between foodborne pathogens and amoebal hosts.

2.5 *Legionella-Acanthamoeba* interactions

The interaction between *Legionella* and *Acanthamoeba* is by far the best studied bacteria-FLA interaction.

2.5.1 Legionella

Legionella pneumophila is a waterborne, Gram-negative, facultative **intracellular pathogen** and the causative agent of Legionellaire's disease - a severe disease with often lethal pneumonia - and Pontiac fever – a milder disease without pneumonia. Most infections can be traced to anthropogenic aquatic systems such as cooling towers and spas, which might produce infected water droplets that are inhaled by people (Borella et al., 2005).

Legionella pneumophila and FLA share the **same aquatic niches** (Declerck et al., 2007; Scheikl et al., 2014; Magnet et al., 2015). Therefore it has been hypothesized that the interaction with free-living protozoa stimulates *Legionella* regrowth and protects the bacteria from stresses - disinfection, chemical and physical - and microbial competition (Storey et al., 2004). Fourteen species of amoebae and two ciliate species have been identified as potential **hosts** of *Legionella.* After intracellular replication, vesicles that contain hundreds of bacteria are produced and released in the environment (Fields, 1996; Fields et al., 2002). The interaction between *L. pneumophila* and *Acanthamoeba* has been extensively studied and striking similarities have been found between the processes by which *Legionella* infects amoeba and mammalian phagocytic cells ((Fields et al., 2002; Escoll et al., 2013), Figure 7). It is therefore suggested that the interaction between *L. pneumophila* and amoebae in the environment provided selective pressure, leading to bacterial factors allowing them to survive also in mammalian macrophages (Steinert et al., 2002).

2.5.2 *Model infection cycle*

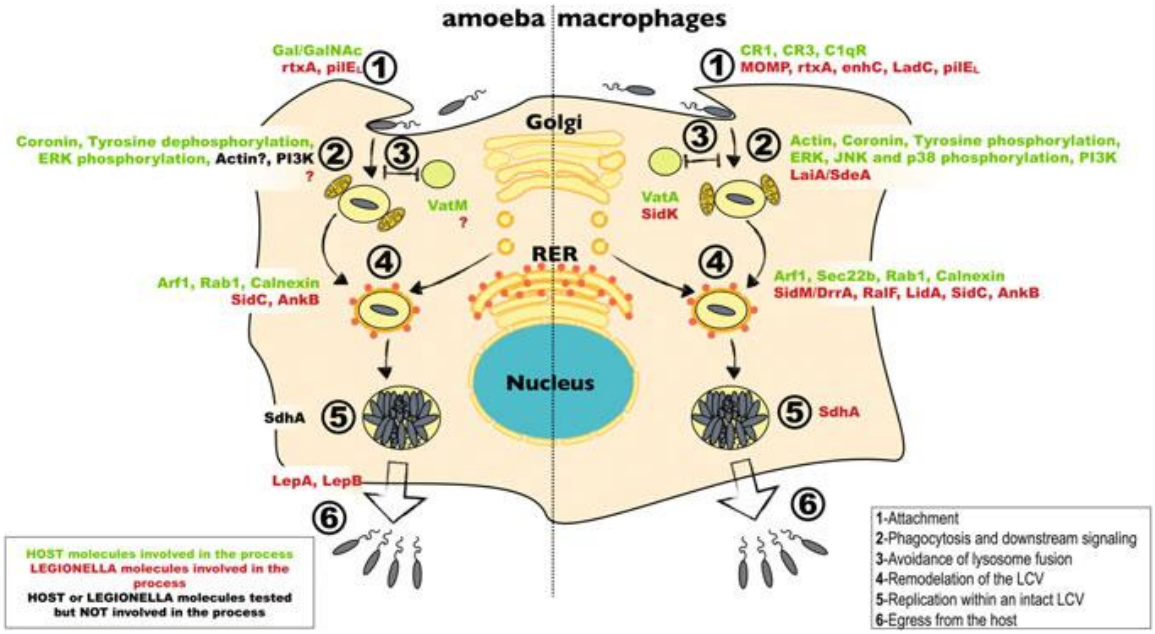


Figure 7: Comparison between a *Legionella* infection in amoeba and macrophages
Figure taken from Escoll et al 2013.

Attachment to the host cells is considered as the first step in the *Legionella* infection cycle. Several bacterial factors have been identified to be essential in the attachment process to both macrophages and amoeba (Escoll et al., 2013). Host-specific factors are also involved in the attachment process. The amoebal Gal/GalNAc lectin receptor is essential for *L. pneumophila* attachment to *Vermamoeba*, but its function is less pronounced in attachment to *Acanthamoeba*, indicating the presence of different attachment mechanisms to different amoebal species. In contrast, attachment to macrophages is mediated by complement receptors on the macrophage surface.

Upon attachment, several **phagocytosis** signaling cascades are activated, including the mitogen-activated protein kinase cascade, Rho GTPases, tyrosine phosphorylases (macrophages) and tyrosine dephosphorylases (amoebae). These pathways trigger coordinated actin polymerization, which induce the formation of plasma membrane protrusions (pseudopods). These pseudopods engulf the bacteria and form a *Legionella* Containing Vacuole (LCV). Both amoebae and macrophages use coiling phagocytosis (unilateral pseudopods), but conventional phagocytosis (bilateral pseudopods) has also been

described (Escoll et al., 2013). The Dot/Icm secretion system (dot: defective in organelle trafficking, icm: intracellular multiplication) is an important virulence factor of *Legionella* that translocates nearly 300 effectors in the host to modulate several signaling and metabolic pathways of the host (Steinert et al., 2002). The Dot/Icm effectors have been suggested to play a role in initial adherence and uptake by macrophages. This role has not yet been confirmed in amoebae. Immediately after *Legionella* uptake, several host cell pathways are activated.

Following phagocytosis, *Legionella* **prevents acidification** of the LCV and **fusion with lysosomes** both in macrophages and amoebae, but by different mechanisms. In macrophages a *Legionella* translocated effector interacts with the vacuolar proton transporter, inhibiting vacuole acidification. In amoebae, *Legionella* prevents the recruitment of an essential transmembrane subunit of the proton transporter to the LCV.

Subsequently, mitochondria and smooth vesicles derived from the host endoplasmic reticulum are hijacked and surround the LCV. Afterwards ribosomes are recruited to the LCV membrane. Several amoeba/macrophage proteins and *Legionella* effectors are involved in these processes, but will not be discussed in detail in this thesis (Figure 7, for a complete overview see (Escoll et al., 2013)). Once the LCV is decorated with ribosomes, non-flagellated *L. pneumophila* commence exponential growth. Growth of Legionellae in amoeba can lead to changes in fatty acid content, outer membrane proteins and lipopolysaccharides (Barker et al., 1993).

During late stages of infection, *Legionella* **exit** the LCV and finish the last rounds of replication within the cytosol. During this stage, bacteria revert to their flagellated infectious form and are released in the environment where they initiate another infection. In macrophages, apoptosis is the main exit-route, though necrosis induced by pore formation has also been described (Alli et al., 2000). In amoeba, necrosis by pore formation occurs (Barker and Brown 1994), but Legionellae can also be expelled in vesicles. It has been shown that such vesicles can contain up to 10E4 bacteria and play an essential role in *Legionella* infections (Berk et al., 1998). Rowbotham (1980) proposed for the first time that humans are infected not by inhaling free legionellae but by inhaling vesicles filled with *Legionella.*

2.5.3 Implications and concerns

Legionella colonizes aquatic systems and can persist for years despite treatments with disinfectants. Several studies show the necessity of amoebae, including *Acanthamoeba* and *Vermamoeba*, for the survival and replication of *L. pneumophila* (Murga et al., 2001; Kuiper et al., 2004). It has been demonstrated that growth within FLA protects the legionellae from adverse environmental conditions (e.g. biocides, disinfectants, (Thomas et al., 2004; Garcia et al., 2007; Dupuy et al., 2014)) and leads to an increased production of polysaccharides and therefore enhance its capacity to establish biofilms (Abdel-Nour et al., 2013). This may explain why eradication of *Legionella* in water systems is difficult.

Furthermore, amoebae have been identified as an important vector or Trojan Horse for transmission of *L. pneumophila* by inhalation of amoebal vesicles/cysts (Brieland et al., 1996; Magnet et al., 2015). Co-inoculation of mice with *Vermamoeba vermiformis* and *L. pneumophila* significantly enhanced intrapulmonary growth of *L. pneumophyla* in mice in comparison to inhalation of legionellae alone (Brieland et al., 1996). Growth of *L. pneumophila* in amoeba enhanced invasion of bacteria in macrophages, and amoebal-grown legionellae were more infectious in mice compared to BCYE agar-grown legionellae (Cirillo et al., 1999).

In addition, cocultivation with *Acanthamoeba* can resuscitate VBNC-stage *L. pneumophila*, which became non-cultivable after disinfection treatment (Garcia et al., 2007; Epalle et al., 2015).

3. Foodborne pathogens and their interaction with free-living amoebae

3.1 Foodborne pathogens: occurrence and impact

Bacterial foodborne pathogens have been identified as the most common source of foodborne zoonotic illness (EFSA, 2015). In Europe, the European Food Safety Authority (EFSA) is responsible for collecting and examining data on zoonoses[7] and foodborne outbreaks[8]. The latter became mandatory since 2005. In the time period 2005-2014, ***Campylobacter jejuni*** was highlighted as the most commonly reported foodborne bacterial pathogen in humans in the European Union (Figure 8). ***Salmonella enterica*** is the second most frequently reported pathogen, followed by ***Yersinia enterocolitica***, **verocytotoxigenic *Escherichia coli*** and ***Listeria monocytogenes***. Various primary production animals carry these bacteria in their gastrointestinal system but are often asymptomatic carriers. Food products can become contaminated during irrigation/fertilization (vegetables) or slaughter and processing (meat). Due to inadequate storage temperatures, insufficient cooking or improper food handling, consumers become exposed to infective amounts of bacterial pathogens.

In humans, foodborne bacterial infections cause gastrointestinal illnesses in general characterized by fever, diarrhea, abdominal cramps, nausea, and sometimes vomiting. Infections are mostly self-limiting, but extra-intestinal infections and complications can occur, especially in people of the YOPI group (Young, Old, Pregnant, Immunocompromised). In order to prevent these diseases, it is important to identify the possible reservoirs and transmission routes.

Free living amoebae and foodborne pathogens share the **same environmental and anthropogenic niches** (Rude et al., 1984; Wildschutte and Lawrence, 2007; Gourabathini et al., 2008; Baré, 2010; Nguyen, 2011; Chavatte et al., 2014). The last decades, it has become clear that FLA can act as a reservoir, vector, shelter, and virulence training ground for

[7] diseases or infections, which are transmissible from animals to humans. The infection can be acquired directly from animals, or through ingestion of contaminated foodstuffs (EFSA)

[8] an incidence, observed under given circumstances, of two or more human cases of the same disease or infection, or a situation in which the observed number of human cases exceeds the expected number and where the cases are linked, or are probably linked, to the same food source' (EFSA, Directive 2003/99/EC).

pathogenic bacteria. Hence, there has been an increasing concern that FLA play a key role in the persistence and transmission of foodborne pathogens.

Unlike the well-studied interactions between *Legionella* and FLA (cf. 2.5), association studies between free living protozoa and foodborne pathogens are scarce. In the following sections, an overview of the current literature reports on the association between the five most commonly reported foodborne pathogenic bacteria (*C. jejuni*, *S. enterica*, *Y. enterocolitica,* verocytotoxigenic *E. coli*, and *L. monocytogenes*) and FLA will be given. Each section will be introduced by a brief description of the pathogenic bacterium itself. An elaborate overview of bacterial transmission in the environment and towards the human host, its infection pattern and associated bacterial virulence and molecular factors involved are out of the scope of this thesis.

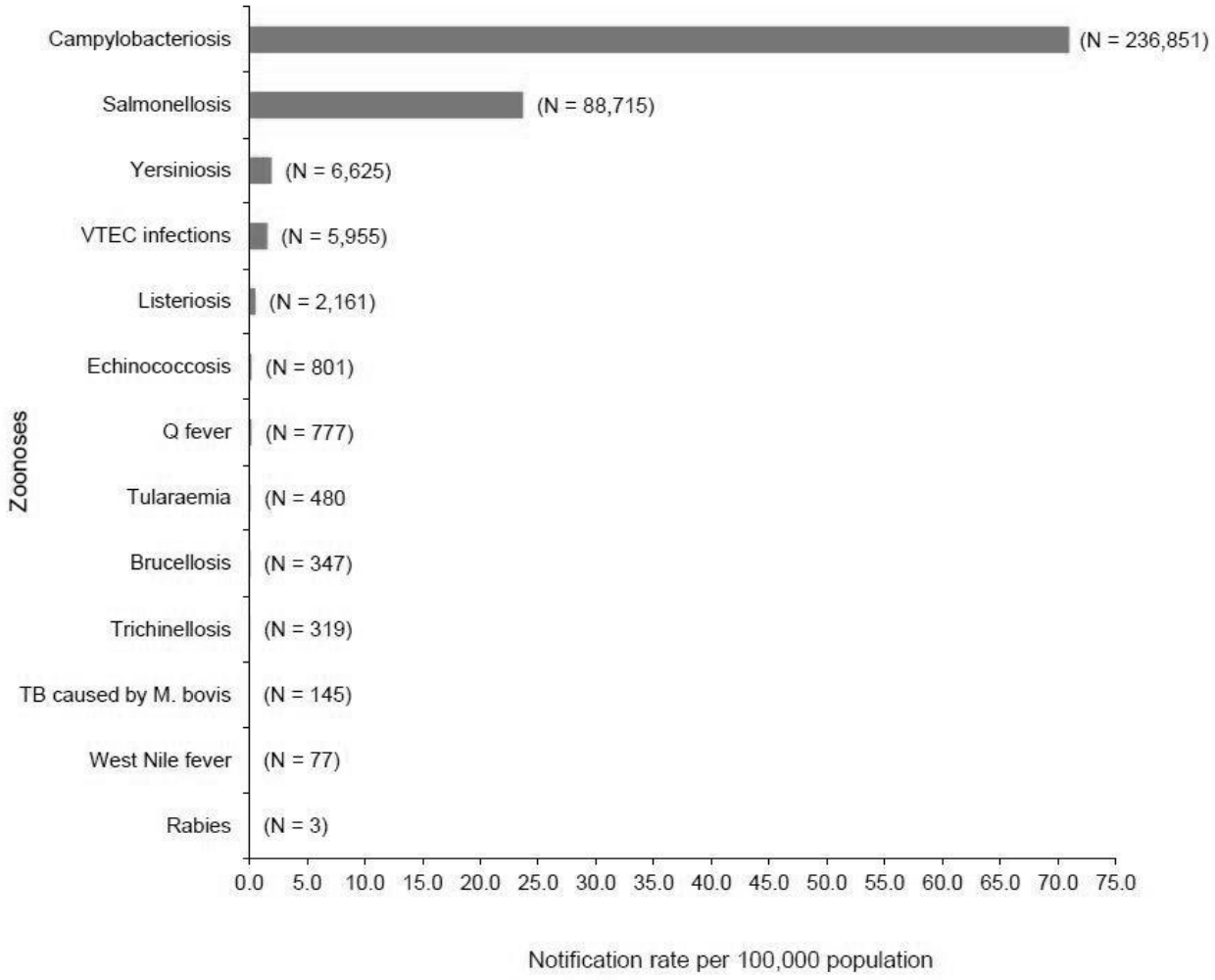


Figure 8: Reported notification rates of foodborne zoonoses in confirmed human cases in the EU in 2014
N: total number of reported cases in EU
Figure taken from ESFA 2015

3.2 Campylobacter jejuni

3.2.1 Characteristics

Campylobacter jejuni is a spiral-shaped, Gram-negative, microaerophilic bacterium, which can be isolated from water and the gastrointestinal tract of poultry, livestock, pets and healthy wildlife. Consumption and handling of raw or undercooked poultry products are considered as the major risk factor for *Campylobacter* infections in humans (EFSA, 2015). *Campylobacter jejuni* can invade the human gut epithelial layer, causing acute, gastrointestinal illness. The most common complication preceded by *C. jejuni* infection is the Guillain-Barré syndrome, a demyelinating disease of the peripheral nervous system resulting in muscle weakness (Parker et al., 2015).

3.2.2 Interaction with FLA

Reports on the interaction and association between *Campylobacte*r and free living protozoa are increasing.

Intracellular survival and multiplication of *C. jejuni* inside *Acanthamoeba* was first described by Axelsson-Olsson (2005). In this study, **intracellular** survival was observed at 4°C, 10°C, 25°C and 30°C, whereas multiplication followed by amoebal lysis occurs at 37°C.

At all tested temperatures, soon after inoculation, *C. jejuni* cells aggregated at certain locations on the *Acanthamoeba* cell wall. After 1 h, live and motile bacteria were seen inside amoebic vacuoles. Internalization was triggered by acidic conditions (Axelsson-Olsson et al., 2010). Campylobacters survived for more than 60 days at 10°C inside *Acanthamoeba* and *C. jejuni* cocultured with amoebae showed a delayed decline in viability compared to bacterial monocultures. Furthermore, a resuscitation of viable but non cultivable bacteria was observed after reinoculation into fresh amoeba cultures (Axelsson-Olsson et al., 2005). Cocultivation with *Acanthamoeba* did not result in detectable genetic changes (Griekspoor et al., 2013).

Campylobacter jejuni are released in the surrounding medium by amoebal lysis (Axelsson-Olsson et al., 2005) or by egestion of vesicles/agreggates (Olofsson et al., 2013; Baré, unpublished).

Amoeba associated campylobacters were more resistant to Virudine® (disinfectant used in poultry industry), chlorine treatment (King et al., 1988) and low pH than planktonic bacteria (Snelling et al., 2005; Axelsson-Olsson et al., 2010). *Campylobacter jejuni* are known to be fragile organisms with reduced ability to tolerate environmental stresses compared to other foodborne pathogens (Park, 2002). By residing inside amoebae, these bacteria escape adverse environmental conditions. Snelling showed that broilers could be colonized by *C. jejuni* residing inside amoeba (Snelling et al., 2008). Intra-amoebal survival was also confirmed by others (Axelsson-Olsson et al., 2005; Snelling et al., 2005; Snelling et al., 2008; Axelsson-Olsson et al., 2010; Baré, 2010; Nguyen, 2011; Olofsson et al., 2013). Other researchers believe *C. jejuni* can **only survive and grow outside amoebae** (Bui et al., 2012; Dirks and Quinlan, 2014). Bui and colleagues reported that *C. jejuni* are rapidly destroyed in amoebal vacuoles under aerobic conditions at 25°C and 37°C. However, under the latter conditions, the number of viable bacteria in the co-culture medium increased significantly over time, indicating that the tested *C. jejuni* strain could survive in coculture with amoebae but not intracellularly. The authors hypothesized that amoebae enhance oxygen depletion in the co-culture medium which is beneficial for the survival and multiplication of *C. jejuni*. The same others also reported that no *C. jejuni* could be seen inside *Acanthamoeba* cysts.

3. 2. 3 Molecular factors involved in the interaction

Transcriptome analysis of *C. jejuni* during the initial interaction stages with *A. castellanii* revealed that oxidative defense genes (such as *katA*, *sodB*, *fdxA*), flagellar genes and unknown genes (Cj0170, Cj1325, Cj1725) were essential in the interaction with *A. castellanii* (Nguyen, 2011).

In addition, Vieira and colleagues recently elucidated almost 50 hypothetical factors, amongst which *FeoB* (ferrous iron transport B), *CmeC* (*Campylobacter* multidrug efflux pump protein) and *CadF* (outer membrane protein) (complete list see Vieira et al., 2015). The analysis was based on orthologous factors identified in other bacterial pathogens or on factors required for *Campylobacter* invasion in macrophages, which share similarities to amoebae. The involvement of these factors still needs to be confirmed experimentally.

3.3 *Salmonella enterica*

3.3.1 Characteristics

Salmonella enterica is a Gram-negative, facultative anaerobic bacillus which consists of six subspecies and over 2500 serovars. The two most frequently reported *S. enterica* serovars in 2014 were *S.* Enteritidis and *S.* Typhimurium (EFSA, 2015). A common reservoir of *Salmonella* is the intestinal tract of domestic and wild animals, which may result in a variety of foodstuffs of both animal and plant origin becoming (in)directly contaminated with faecal material (EFSA, 2015). Many human infections are due to ingestion of contaminated food, especially eggs, poultry meat and pork. *Salmonella enterica* can colonize the human intestines and may penetrate into the intestinal epithelium. Infections are mostly self-limiting, though complications such as dehydration and septicemia may occur (Engab et al., 2015).

3.3.2. Interaction with FLA

Salmonella have been recovered from environmental *Acanthamoeba,* indicating that amoebae act as a reservoir and vector for *Salmonella* spp. (Hadas et al., 2004). Anacarso reported **intracellular growth** of *S. enterica* serovar Enteritidis in *A. polyphaga* with 2 log increase after 72h (Anacarso et al., 2011). Intra-amoebal salmonellae are localized within membrane-bound contractile vacuoles in *Acanthamoeba* in which they can survive for at least 4 days and multiply to up to 200 cells per vacuole (Gaze et al., 2003). Though uptake and intracellular replication in *Acanthamoeba* is also confirmed by others (e.g.Tezcan-Merdol et al., 2004; Feng et al., 2009; Akya et al., 2010), several *Salmonella* serovars/strains are digested or seem to have an exclusively extracellular association with amoeba under certain environmental conditions (Gaze et al., 2003; Huws et al., 2008). Douesnard-Malo assessed that *S. enterica* serovar Typhi survived cocultivation with *A. castellanii* for at least 3 weeks, as opposed to less than 10 days when grown in monoculture. The salmonellae surviving in coculture were not intracellular and did not require a physical contact with amoebae for their prolonged survival (Douesnard-Malo and Daigle, 2011).

Wildschutte (2004) reported that different *S. enterica* serovars are grazed by intestinal amoebae at different rates, indicating that amoebae recognize the O-antigen at the bacterial cell surface and take up the bacteria with different efficiencies (Marciano-Cabral et al., 2003).

The **gene melting pot** hypothesis (cf. 2.4) was confirmed by McCuddin (2006) who proved transfer of the ceftriaxone resistance gene from *Klebsiella* to *Salmonella* occurring in rumen protozoa. Moreover cultivation with rumen protozoa enhanced invasion and was related to a rapid onset and progression of the disease in cattle (Rasmussen et al., 2005; Xiong et al., 2010).

Contradictory reports can be found on the **cytotoxicity** of *Salmonella* on amoebae. Intracellular replication is followed by an apoptosis-like cell death (at 24h post infection, p.i.) in infected *Acanthamoeba* (Feng et al 2009). Detachment and killing of amoebae after cultivation (16h p.i.) with *Salmonella* was also described by Tezcan-Merdol (2004). In contrast, no cytotoxic effect of the bacteria on the amoebae was observed by Douesnard-Malo (2011). Differences in cytotoxic behavior can be attributed to amoebal/bacteria species and strain differences, and to variations in environmental conditions. Gaze (2003) reported that *S. enterica* serovar Typhimurium could also be released in fecal pellets of amoeba, though this could not be confirmed by Gourabathini (2008) who used *S. enterica* serovar Thompson.

3.3.3 Molecular factors involved in the interaction

Several *S. enterica* factors have been shown to be important in the interaction with amoebae.

Salmonella pathogenicity island 2 (SPI2), which encodes a Type III secretion system (T3SS), and the *Salmonella* virulence plasmid are essential in the survival of *S. enterica* serovar Typhimurium in *Acanthamoeba* (Tezcan-Merdol et al., 2004; Bleasdale et al., 2009). The SPI2-T3SS is also required for *Salmonella* survival in macrophages, where it is involved in translocation of bacterial effector proteins in the host macrophages. Feng and colleagues (2009) confirmed that genes from SPI2 and the virulence plasmid were upregulated inside

amoeba. Also the transcriptional regulator FIS and SPI1-genes were up-regulated, while flagella genes of salmonellae were down-regulated (Gaze et al., 2003; Feng et al., 2009).
PhoP, a DNA binding protein associated with regulation of many genes including the SPI2-encoded T3SS genes, is also essential for survival inside *Acanthamoeba* (Bleasdale et al., 2009).

3.4 *Yersinia enterocolitica*

3.4.1 Characteristics

Yersinia enterocolitica is a Gram-negative, facultative anaerobic, psychrotolerant bacillus. Based on biochemical characteristics and lipopolysaccharide O-antigens, the species is divided in 6 biotypes and over 70 serotypes. Bioserotypes 2/O:9 and 4/O:3 are amongst the most frequently reported pathogenic *Y. enterocolitica* in Europe (EFSA, 2015). Gastrointestinal infections are mainly correlated with the consumption of (raw) pork. Pigs are considered as a main reservoir, carrying pathogenic *Yersinia* in their tonsils and gastrointestinal system. Common symptoms of *Y. enterocolitica* infection are acute gastroenteritis and pseudoappendicitis (Drummond et al., 2012).

3.4.2 Interaction with FLA and molecular factors involved

Data about the association of *Y. enterocolitica* with free-living protozoa is scarce. In 1988, King proved that bacteria, amongst which *Y. enterocolitica*, became more resistant against chlorine treatment after association with *Acanthamoeba* (King et al., 1988). Another study reported early intracellular replication, 8 up to 24 h p.i. in *Acanthamoeba polyphaga*, followed by killing and digestion (Anacarso et al., 2011).

3.4.3. Molecular factors involved in the interaction

There are no reports of genes or molecules that could be involved in the interaction of *Y. enterocolitica* with FLA.

3.5 Verocytotoxigenic *Escherichia coli*

3.5.1 Characteristics

Escherichia coli is a Gram-negative, facultative anaerobic bacillus and a commensal inhabiting the intestinal tract of humans and animals. Some strains however, such as enterohaemorrhagic *E. coli* (EHEC) can cause severe foodborne disease. Cattle are the main reservoir for EHEC, especially serotype O157:H7. Transmission to humans mainly occurs through consumption of contaminated food e.g. bovine meat, raw milk and raw vegetables (EFSA, 2015). The EHEC strains comprise a subgroup of the STEC group (Shiga Toxin producing *E. coli,* also called VTEC, Verocytotoxigenic *E. coli*).

Clinical symptoms may include bloody diarrhea and hemorrhagic colitis, along with complications such as haemolytic uraemic syndrome (HUS), characterized by acute renal failure, haemolytic anaemia and thrombocytopenia (Croxen et al., 2013).

3.5.2 Interaction with FLA

Verocytotoxin-producing *Escherichia coli* 0157:H7 survived and **replicated in** *A. polyphaga* (Barker et al., 1999). Microscopical analysis revealed that several trophozoites contained ten or more *E. coli* cells within membrane bound vacuoles and that only in some of them bacterial digestion took place. In general, cocultivation with amoebae resulted in higher bacterial numbers (Barker et al., 1999) and in less bacteria shifting into a VBNC state compared to bacterial monocultures (Chekabab et al., 2012). The latter authors also noted a reduced amoebal growth rate, and an increased amoebal mortality, mediated by *E. coli* Shiga toxins in the cocultures.

Other cocultivation studies performed with non-verocytotoxin producing *E. coli* strains, such as the enteropathogenic *E. coli* O127:H6 and the nonpathogenic K-12 strain showed that these strains did not survive predation by *Acanthamoeba* nor had a toxic effect on the amoeba (Huws et al., 2008; Chekabab et al., 2012).

3.5.3. Molecular factors involved in the interaction

Chekabab and others (2012) showed that the Pho regulon was required for *E*. *coli* O157:H7 growth when cocultured with *A*. *castellanii*, whereas shiga toxins as such were not essential for bacterial survival in cocultures.

The Pho regulon allows bacteria to adapt to low inorganic phosphate concentrations and is also known to regulate bacterial virulence. The exact mechanisms by which the Pho regulon enhances intra-amoebal survival is not known yet. Other factors such as the outer membrane protein A (OmpA), lipopolysaccharide (LPS) and the T3SS are critical bacterial determinants involved in uptake/invasion and survival of the neuropathogenic *E.coli* K1 within *Acanthamoeba* (Jung et al., 2007; Siddiqui et al., 2011). Although the requirement of these factors has not been confirmed for VTEC, it is likely that they interact by similar mechanisms. Transcriptional profiling of *E. coli* O:175:H7 within *A. castellanii* revealed that 655 genes were upregulated, including shiga toxin genes (stx1A, stx1B stx2A), genes involved in T3SS components, and SOS response genes such as recA (Carruthers et al., 2010).

3.6 *Listeria monocytogenes*

3.6.1 Characteristics

Listeria monocytogenes is a Gram-positive, facultative anaerobic, non-sporulating, psychrotolerant bacillus. The bacterium is widely distributed in the environment where it exhibits a saprophytic lifestyle in e.g. soil, water and sewage. No environmental host reservoir has been identified so far (Schuppler, 2014). Human infections occur most likely due to ingestion of contaminated food. A broad range of foodstuffs can be contaminated. Fish products and refrigerated ready-to-eat food products are particularly of concern as storage for extended times under cold temperatures allows the psychrotolerant *Listeria* to grow, while growth of other competing microorganisms is inhibited (Chan and Wiedmann, 2009). Human gastroenteritis caused by *L. monocytogenes* is mostly self-limiting. However, in immunocompromised people more severe conditions including septicemia and encephalitis are encountered. In pregnant women, complications such as spontaneous abortion and stillbirth can occur (Drevets and Bronze, 2008).

3.6.2 Interaction with FLA

There have been a number of reports on the interaction between *Listeria monocytogenes* and free living amoeba with somewhat contradictory results.
Whilst some authors claim that they clearly observed **intra-amoebal** survival (Anacarso et al., 2011), others are convinced that *L. monocytogenes* can only survive **extracellularly** and are rapidly digested once entering the food vacuoles (Schuppler, 2014).

An early study showed that *L. monocytogenes* survived phagocytosis by *Acanthamoeba*. After 8 days the amoeba started to encyst and some of the amoebae were ruptured, releasing viable *Listeria* into the environment (Ly and Muller, 1990). Zhou and coworkers (2007) confirmed that the bacteria survive predation by *A. castellanii* for at least 72h, but they found no evidence for intra-amoebal replication. However, intracellular multiplication by 4 log after 72h cocultivation with *A. polyphaga* was observed by Anacarso (2011).

In contrast, other authors reported that *L. monocytogenes* only grows extracellularly due to metabolic waste products released during co-culture, and that there is no intra-amoebic replication (Zhou et al., 2007; Huws et al., 2008; Akya et al., 2009, 2010; Fieseler et al., 2014). Uptake followed by rapid intracellular digestion was clearly demonstrated by Doyscher and coworkers (2013). They also observed a peculiar type of bacterial uptake, whereby *L. monocytogenes* cells were first assembled into large aggregates of densely packed bacteria (**'backpacks'**) on the surface of the *Acanthamoeba* cells. The backpacks were carried along, and were eventually phagocytized.

3.6.3 Molecular factors involved in the interaction

Little is known about the molecular mechanisms involved in *L. monocytogenes* uptake, intra-amoebal trafficking and digestion. Zhou and coworkers (2007) suggested that listeriolysin O might contribute to improved survival inside *Acanthamoeba* as mutant strains exhibited poorer survival and were not able to escape the phagosome. The pore forming toxin listeriolysin O is considered as a major virulence factor and facilitates intracellular survival in macrophages (Arnett et al., 2014).

3.7 Association with FLA: implications and concerns for food safety and public health

Despite strict surveillance and control measures, foodborne illnesses continue to rise across Europe and outbreaks of major public health significance still occur. The last decades, increasing attention has been paid to the interactions between foodborne pathogenic bacteria and free-living amoebae as both are commonly encountered in/on food and in food related environments. Is has become clear that amoebae are a potential **reservoir, vector, shelter, training ground and gene melting pot** for several important foodborne pathogens (summary Table 1). As such, there is a growing concern that FLA are involved in the contamination and persistence of foodborne pathogens in food related environments.

Foodborne pathogens such as *C. jejuni*, *S. enterica*, *E. coli* and *L. monocytogenes* are able to multiply in or in association with FLA (Vaerewijck et al., 2014). This may lead to an increase in bacterial numbers. Furthermore, internalized bacteria are easily dispersed in the environment by motile amoebae or through packaging in airborne vesicles or cysts. Moreover, intra-amoebal bacteria are protected against antimicrobial agents, and an increased virulence after passage through a protozoan has been observed for several bacteria (Cirillo et al., 1999, Rasmussen et al., 2005, Xiong et al., 2010).These observations underscore the significance of FLA in pathogen persistence and imply a potential risk for food safety and public health.

Currently, microbial food safety monitoring focusses on detection and enumeration of pathogens and indicator organisms. Free-living protozoa are not included in these standardized tests as they are considered as harmless and are unknown to many quality control managers, food producers and consumers. Noteworthy, it has been shown that bacterial associations with FLA may hamper tests to quantify (pathogenic) bacteria (Dietersdorfer et al., 2016). Most of these tests are based on Colony Forming Units (CFU) on agar. It has been demonstrated that bacteria inside amoebae and vesicles/pellets are clustered and difficult to disperse. Hence, one CFU may result from multiple bacteria inside one vesicle, leading to a underestimation of the real number of bacteria (Berk et al., 1998). So, the presence of FLA on/in food and on food related environments should no longer be ignored.

	FLA can act as				Survival in cysts	Beneficial extracellular association
	Reservoir	Vector	Shelter	Training ground/ cross resistance		
Campylobacter jejuni	V (Axelsson-Olsson 2005, 2013, Snelling 2005,2008, Nguyen 2011, Olofsson 2013)	V (Snelling 2005, 2008)	V (King 1988, Snelling 2005, Axelsson-Olsson 2010)			V (Snelling 2005, Baré 2010, Bui 2012)
Salmonella enterica	V (Gaze 2003,Hadas 2004, Anacarso 2011)	V (Gaze 2003, Hadas 2003)	V (Brandl 2005)	V (Rasmussen 2005,McCuddin 2006, Xiong 2011)		V (Gaze 2003, Huws 2008, Bui 2012, Douesnard-Malo 2011)
Yersinia enterocolitica			V (King 1988)			
Verocytotoxigenic *Escherichia coli*	V (Barker 1999, Chekabab 2012)				V (Matin 2011)	
Listeria monocytogenes	V (Ly and Muller 1990, Anacarso 2011) X (Zhou 2007, Huws 2008, Akya 2009,2010, Fieseler 2014)			V	X (Ly and Muller 1990)	V (Huws 2008, Zhou 2007, Fieseler 2014)

Table 1 Overview of major studies that confirmed interactions between FLA and several foodborne pathogens.

All studies were performed *in vitro* unless indicated otherwise. *in situ* study, *in vivo* study

V: tested and advantageous role of FLA confirmed, X: tested but not confirmed

reservoir: the amoeba act as a host, wherein bacteria are able to grow (Brown and Barker, 1999)

vector: the amoeba act as a vehicle which actively (trophozoites) or passively (cyst) spreads the internalized bacteria (Anacarso et al., 2011)

shelter: the amoeba can protect the internalized bacteria against stressfull environmental conditions

training ground/cross resistance: the amoeba can increase bacterial virulence or increase persistence against stressfull conditions (Molmeret et al., 2005)

beneficial extracellular association: bacteria benefit from an extracellular amoeba association (eg. by using secreted amoebal metabolites)

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CHAPTER II

Aims

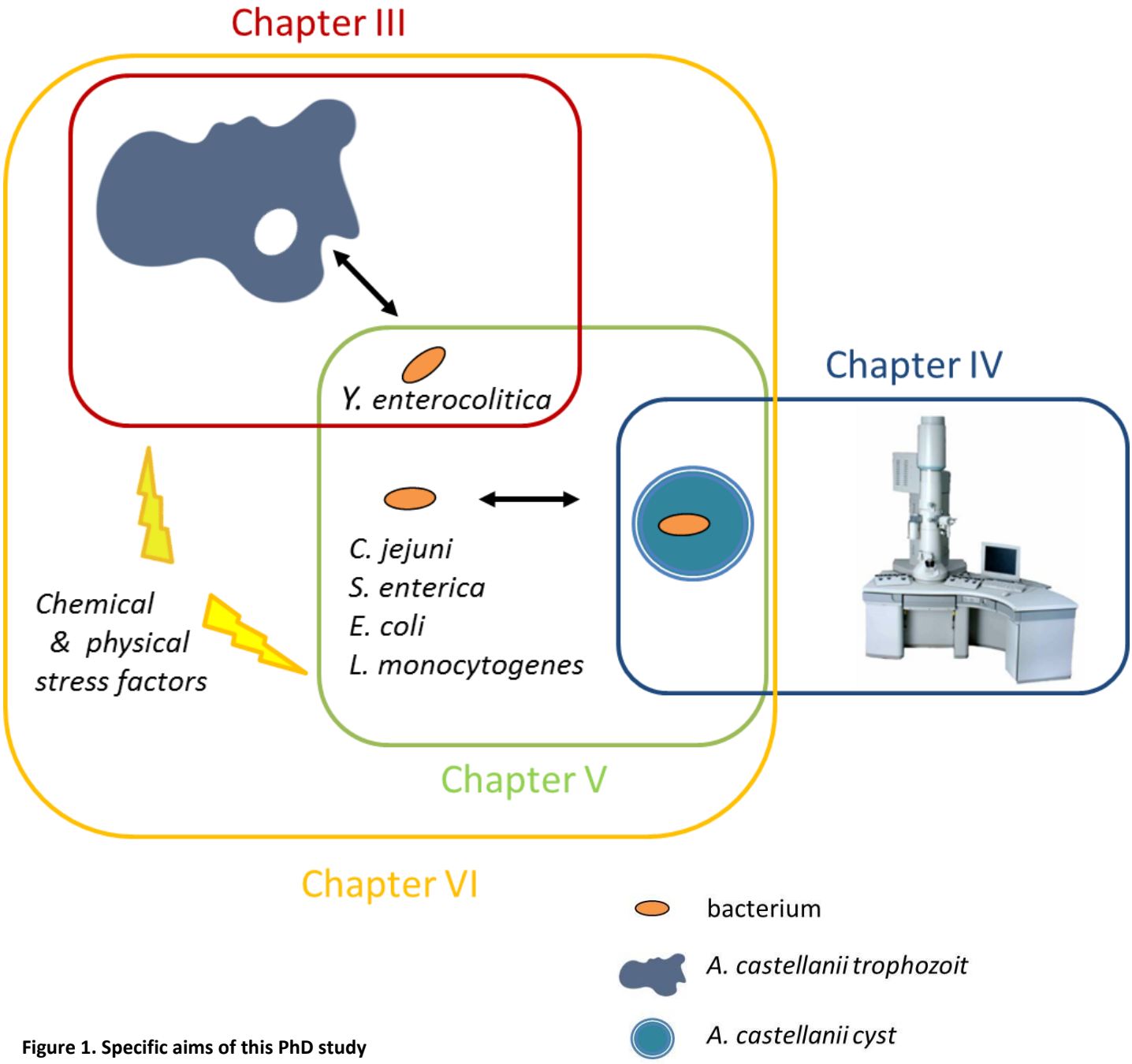

Figure 1. Specific aims of this PhD study

The foodborne pathogenic bacteria *Campylobacter jejuni*, *Salmonella enterica*, verocytotoxigenic *Escherichia coli, Yersinia enterocolitica* and *Listeria monocytogenes* are among the most frequently reported causes of foodborne illness. Despite thorough disinfection protocols and hygiene monitoring during food processing, these bacteria often persist in food-related environments and on food.

As Free Living Protozoa (FLP) may act as a reservoir, vector, protective hosts and virulence training ground for bacteria, there is a growing concern that they may play a role in the, transmission and persistence of pathogenic bacteria in food-related environments. The few studies which have already indicated protozoal grazing/digestion resistance of *C. jejuni*, *S. enterica*, *E. coli* and *L. monocytogenes*, strengthen this concern.

The general aim of this doctoral research was to study the interactions between the free-living, ubiquitous model amoeba, *Acanthamoeba castellanii* and the foodborne bacterial pathogens *C. jejuni*, *S. enterica*, *Y. enterocolitica*, *E. coli* and *L. monocytogenes.*

To this end, the specific goals of this study were (Figure 1):

- ✓ Assess the impact and the nature of the associations between *Y. enterocolitica* and free-living protozoa by coculture assays, and more specifically the ability of the bacterial pathogen to grow/survive intra or extracellular of the amoebal trophozoit under various environmental conditions (CHAPTER III).
- ✓ Evaluation of methodologies for detection of pathogenic foodborne bacteria inside robust amoebal cysts, with the development and testing of various sample preparation protocols for Transmission Electron Microscopy (CHAPTER IV).
- ✓ Determine the uptake/invasion efficiency, long-term intracystic survival capacities and intracystic location of the *C. jejuni*, *S. enterica*, *Y. enterocolitica*, *E. coli* and *L. monocytogenes* (CHAPTER V)
- ✓ Evaluate the tolerance of amoebal cysts, trophozoites and associated foodborne bacteria against stressful chemical and physical conditions frequently used in domestic and industrial food related environments (CHAPTER VI).

CHAPTER III

Yersinia enterocolitica behavior in the presence of the bacterivorous *Acanthamoeba castellanii*

Chapter redrafted after:

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1. Abstract

Free-living protozoa may play an underestimated role in the ecology and epidemiology of human pathogenic bacteria. In the present study, the interaction between *Yersinia enterocolitica*, an important foodborne pathogen, and the free-living amoeba *Acanthamoeba castellanii* was studied. Several *in vitro* cocultivation assays were set up to assess the resistance of *Y. enterocolitica* to *A. castellanii* predation and the impact of environmental factors and bacterial strain-specific characteristics. Results showed that all *Y. enterocolitica* strains persist in association with *A. castellanii* for at least 14 days, and association with *A. castellanii* enhanced survival of *Yersinia* under nutrient-rich conditions at 25 °C (+ 2.4 Log_{10} after 14d) and under nutrient-poor conditions at 37 °C (+ 1 Log_{10} after 14d). Amoebae cultivated in the supernatant of one *Yersinia* strain showed a temperature- and time-dependent permeabilization. Intraprotozoan survival of *Y. enterocolitica* depended on nutrient availability and temperature, with up to 2.8 log cfu/mL bacteria displaying intracellular survival at 7 °C for at least 4 days in nutrient-rich medium. Transmission electron microscopy was performed to locate the *Yersinia* inside the amoebae.

2. Introduction

Yersinia enterocolitica is the third most frequently reported foodborne pathogen in the European Union, with 7017 reported human cases in 2011 (EFSA, 2013). Yersiniosis occurs mainly in infants and preschoolers and is characterized by acute enteritis with fever and diarrhea. In adolescents and adults, pseudo-appendicitis, sequelae as arthritis and erythema nodosum, and septicemia can also occur (Verhaegen et al., 1998; Bottone, 1999). Most infections are caused by *Y. enterocolitica* bioserotypes 4/O:3 and 2/O:9, which all harbor a virulence plasmid (pYV) that is required for full pathogenicity in humans (Bottone, 1997; EFSA, 2013). This virulence plasmid is optimally expressed at 37 °C, but can become lost during laboratory cultivation (Riley and Toma, 1989; Farmer et al., 1992).

Most *Y. enterocolitica* infections in humans are associated with the handling and consumption of raw or undercooked pork products (EFSA, 2013). Pigs are considered as the primary reservoir of human pathogenic *Y. enterocolitica* as they harbor these pathogens in the tonsils and gastro-intestinal lymphoid tissue (Nesbakken et al., 2003; Van Damme et al., 2010). However, many factors related to the epidemiology of *Y. enterocolitica*, such as environmental sources and transmission routes, remain indistinct (Fredriksson-Ahomaa et al., 2006) .

Free-living protozoa are unicellular, heterotrophic, eukaryotic micro-organisms ubiquitous in natural and anthropogenic aquatic and terrestrial habitats, including food-related environments (Vaerewijck et al., 2008; Baré et al., 2009; Vaerewijck et al., 2010; Baré et al., 2011). Free-living protozoa, including members of the amoebal genus *Acanthamoeba* graze on bacteria and are therefore considered as important bacterial predators. However, various bacteria are able to evade protozoan ingestion and/or digestion and can even benefit from the association with protozoa (Matz and Kjelleberg, 2005). Some bacteria enhance their survival by extracellular association (Akya et al., 2009), though others are taken up and enter a membrane bound vacuole, resisting protozoan digestion (Greub and Raoult, 2004; Whitworth et al., 2005; Berk et al., 2008). Both associations result in survival, and even multiplication, of the bacteria in the presence of its predator. The intraprotozoan location further represents a shelter for bacteria against physical and chemical environmental

conditions (King et al., 1988; Berk et al., 2008). Moreover, free-living protozoa may serve as a vehicle for the colonization of new habitats (Berk et al., 2008) and hosts (Snelling et al., 2008). Free-living protozoa can also play a role in the selection of bacterial virulence traits and adaptation to survival in macrophages (Cirillo et al., 1994; Cirillo et al., 1997; Bruggemann et al., 2006; Koubar et al., 2011). In addition, gene transfer between intraprotozoan bacteria and protozoan host has been reported (McCuddin et al., 2006)

Yersinia enterocolitica and free-living protozoa share the same ecological niches, such as water (Hausmann et al., 2003; Falcão et al., 2004), vegetables (Cocolin and Comi, 2005; Vaerewijck et al., 2011) and anthropogenic environments, e.g. domestic refrigerators (Jackson et al., 2007; Vaerewijck et al., 2010). The role of free-living protozoa in the ecology and epidemiology of foodborne pathogens, such as *Campylobacter, Salmonella* and *E. coli* has already been documented (Alsam et al., 2006; Axelsson-Olsson et al., 2010a; Douesnard-Malo and Daigle, 2011), but for *Y. enterocolitica,* only a brief description of an intraprotozoan replication, probably followed by the killing and digestion of the bacterial strain (Anacarso et al., 2011), and an increased resistance to free chlorine in their early intraprotozoan lifestyle (King et al., 1988) have been reported so far.

To further elucidate the ecology and epidemiology of this common pathogen, evaluation of the importance of its association with free-living protozoa, and the influence of environmental factors and *Yersinia* strain characteristics thereon, is needed.

The objectives of the present study were to assess the impact and the nature of the associations between *Y. enterocolitica* and free-living protozoa. Therefore, *in vitro* coculture experiments using the model protozoon *Acanthamoeba castellanii* and *Yersinia* strains with different bacterial characteristics were performed under different environmental conditions. These coculture experiments (i) evaluated the ability of *Y. enterocolitica* to survive or grow in association with the bacterivorous *Acanthamoeba* under different conditions, (ii) assessed if the yersiniae could survive or grow extracellularly on factors released by the amoebae and *vice versa*, and (iii) investigated if *Y. enterocolitica* were resistant to amoebal digestion, if they could survive or grow intracellularly, and where they were exactly located inside *A. castellanii*.

3. Materials and methods

3.1 Cultivation of *Acanthamoeba castellanii*

Acanthamoeba castellanii (genotype T4, American Type Culture Collection ATCC30234, isolated from yeast culture, 1930) was maintained axenically in Proteose peptone Yeast extract Glucose medium (PYG[1], ATCC-recipe, http//www.lgcstandards-atcc.org) at 25 °C in 75 cm2 culture flasks. Light microscopic observations and plating of culture samples on Plate Count Agar (PCA, Biorad, Hercules, California, USA), which were incubated at 30 °C for 48 h, were performed to verify axenicity of the culture. For coculture experiments, stationary growth phase amoebae (3.5 days old) forming a monolayer were used. The adherent amoebae were harvested by tapping the flasks and subsequent centrifugation of the solution (300 x g, 5 min), washed with Page's Amoeba Saline (PAS[2], ATCC-recipe), and resuspended in PYG or PAS medium, depending on the experiment to be performed. For further interaction studies, the *Acanthamoeba* culture was diluted to *c.* $5x10^5$ living trophozoites/mL. The actual cell numbers were determined with a Füchs-Rosenthal chamber (Blaubrand, Wertheim, Germany). The membrane integrity and thus also the cell viability of the *Acanthamoeba* trophozoites was assessed using the trypan blue exclusion assay (Baré et al., 2010).

3.2 Cultivation of *Yersinia enterocolitica* strains.

Four *Y. enterocolitica* strains, isolated between 2010-2011, were used in this study: strain YeH3+ (bioserotype 4/O:3, pYV+), and its pYV-plasmid cured[3] derivate YeH3- were isolated from the stool of a one-year old boy, strain YeH9- (bioserotype 2/O:9, pYV-) from the stool of a 43-year old woman, and strain YeM3+ (bioserotype 4/O:3, pYV+) from minced pork. All strains were maintained in glycerol at -20 °C. Strains were cultivated in Tryptone Soya Broth (TSB, Biorad, Hercules, California, USA) at 37 °C for 24 h to activate the pYV-virulence

[1] 20 g proteose peptone, 1 g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$, 1 g sodium citrate $\cdot 2H_2O$, 0.02 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 1.7 g KH_2PO_4, 0.34 g $Na_2HPO_4 \cdot 7H_2O$, 9 g dextrose, and 0.059 g $CaCl_2$ in 1 liter of distilled water

[2] 120 mg NaCl, 4 mg $MgSO_4 \cdot 7H_2O$, 4 mg $CaCl_2 \cdot 2H_2O$, 142 mg Na_2HPO_4, and 136 mg KH_2PO_4 in 1 liter of distilled water

[3] due to long-term cultivation at 30 °C.

plasmid (Rohde et al., 1999). At the stationary growth phase, bacteria were harvested by centrifugation (10,000 x g, 5 min), washed in PAS and resuspended in PYG or PAS (depending on the experimental setup). For use in further experiments, the bacterial suspension was diluted to *c.* $5x10^7$ cfu/ml by using data from *Y. enterocolitica* growth curves. The exact number of viable bacteria was determined after plating a serial dilution of the suspension on PCA and 48 h incubation at 30 °C. Before the start of the experiments and after each coculture experiment, the bioserotype and the presence of the virulence plasmid of each *Y. enterocolitica* strain were verified by biochemical testing and PCR analysis as described by Van Damme (2013).

3.3 Coculture experiments

Coculture experiments with *A. castellanii* and the four *Y. enterocolitica* strains were performed in both nutrient-rich (PYG) and nutrient-poor (PAS) media (Axelsson-Olsson et al., 2010b; Baré et al., 2010; Anacarso et al., 2011) at 7, 25 and 37 °C. Those nutrient and temperature conditions were used to mimic natural (e.g. aquatic areas (Berry et al., 2010)), food-related environmental conditions (eg. refrigerators, temperatures and organic loads in food processing areas (Vaerewijck et al., 2010; Vaerewijck et al., 2012)), and the mammal body temperature.

3.4 Persistence of *Yersinia* in coculture with *Acanthamoeba castellanii*

The ability of *Y. enterocolitica* to survive in association with the bacterivorous amoebae was assessed by persistence assays. With these assays, no distinction could be made if recovered bacteria were internalized in the amoebae or if it concerned adherent or free-living extracellular bacteria. *Acanthamoeba castellanii* cells were seeded into 12-well plates (2 mL/well at a concentration of *c.* $5x10^5$ living trophozoites/mL PYG or PAS) and plates were incubated at 25 °C for 2 h to allow amoebal settlement and adhesion. Then, the medium was gently removed and replaced by 2 mL *Y. enterocolitica* suspension (*c.* $5x10^5$ or $5x10^7$ cfu/mL; dissolved in PYG or PAS) to achieve a multiplicity of infection of *c.*1 bacteria per amoeba and *c.*100 bacteria per amoeba, respectively. Plates were centrifuged at 50 x g for 5 min to

enhance cell contact and incubated at 7, 25 or 37 °C. Monocultures of *A. castellanii* and of each *Yersinia* strain were included as controls. At day 1, 2, 3, 6, 7, 10 and 14, the cells were harvested by scraping the wells. The amoebal cell integrity was visually evaluated by light microscopy and the trypan blue exclusion assay. The number of viable *Yersinia* was determined after plating serial dilutions of the cell suspension on PCA and incubation for 48 h at 30 °C.

3.5 Effect of released factors on microbial survival and growth

The ability of *Y. enterocolitica* to survive and/or grow in association with *A. castellanii* without direct interaction with the amoebae, i.e. extracellularly on factors released by the amoebae, and vice versa, was determined by supernatant assays. Therefore, *A. castellanii* (c. 5x 10^5 cells/mL) and *Yersinia* strains (c. 5x 10^7 cfu/mL) were harvested after 5 min centrifugation at 300 x g and 5 min at 10,000 x g, respectively. The supernatant of both the bacterial and amoebal monocultures was filter-sterilized (0.22 µm, Nalgene Syringe cellulose acetate membrane, Thermo Scientific, Langenselbold, Germany) and the pH of the bacterial supernatant was measured (WTW pH 330i, WTW, Weilheim, Germany). Subsequently, the *Yersinia* were resuspended in the supernatant of the amoeba cultures and *vice versa*. One mL of each *Yersinia* or *A. castellanii* suspension was inoculated in 12 well-plates. As controls, amoebae suspended in TSB-medium (growth medium of the *Yersinia* cultures) and *Y. enterocolitica* strains suspended in PYG-medium (growth medium of *Acanthamoeba*) were included. All plates were incubated at 7, 25 and 37 °C. After 3 h and 1, 2 and 3 days of incubation, cells were harvested by scraping the wells. The number of viable *Yersinia* and the amoebal concentration and cell integrity were determined as described above.

3.6 Intraprotozoan survival of *Y. enterocolitica*

The ability of *Y. enterocolitica* to survive inside *A. castellanii*, and the enumeration of the intracellular *Yersinia,* was assessed by gentamycin protection assays**.** Cocultures and amoebal monocultures were set up as described above for the persistence assays. After 2 h of (co)cultivation, the medium was gently removed and the cells were washed with PAS to

minimize extracellular bacteria. Afterwards, 4 mL of gentamycin sulphate solution (Sigma-Aldrich, St. Louis, USA) at a final concentration of 100 µg/mL PYG (Anacarso et al., 2011) was added to each well and the plates were incubated for 1 h at 7, 25 or 37 °C to kill the extracellular *Y. enterocolitica.* In previous tests, this gentamycin treatment was effective in killing 99.97 to 100% of the *Y. enterocolitica*, with no effect on the viability of *Acanthamoeba castellanii* (data not shown). For the experiments in PAS, also gentamycin suspended in PYG was used for initial killing of the extracellular bacteria, as this treatment was most effective (Anacarso et al., 2011). After incubation, wells were washed with PAS and 4 mL gentamycin maintenance solution (20 µg gentamycin/mL PAS or PYG, depending on the experimental setup) was added to each well. This moment was defined as the 0 h-time point (*i.e.* 3 h after initial setup of the (co)culture). The plates were further incubated at 7, 25 or 37 °C, and after 1, 2, 3 and 4 days the number of amoebae and the intra- and extra-amoebal bacterial counts were determined. For the extra-amoebal bacterial counts, the supernatant was removed and plated on PCA plates. Afterwards wells were washed with PAS, 4 mL PYG or PAS was added to each well and the amoebae were harvested by cell scraping. This cell suspension was subsampled for further analysis: 0.5 mL was used for enumeration and viability testing of amoebae as described above, 2 mL of the cell suspension was used for transmission electron microscopy analysis (*cf*. below), and the remaining amoebal suspension (1.5 mL) was used to determine the intra-amoebal bacterial counts. For the latter, the amoebae were lysed with 0.5% sodiumdeoxycholate for 5 min at room temperature, which has been shown to result in effective lysis of the amoebae without affecting intracellular bacteria (data not shown). The suspension was plated on PCA plates. All plates were incubated at 30 °C for 48 h, after which the extra- and intracellular *Yersinia* were counted.

3.7 Localization of intraprotozoan *Y. enterocolitica*

Transmission electron microscopy (TEM) was applied to determine the exact subcellular location of intraprotozoan *Y. enterocolitica*. The amoebae from the coculture and control wells from the gentamycin protection assays (second aliquot) were harvested by centrifugation at 300 x g for 5 min and serially fixed with glutaraldehyde in sodium cacodylate buffer (pH 7.4) as follows: 0.8%, 1.25% and finally 2.5% glutaraldehyde in 0.05M

sodium cacodylate buffer (each concentration includes 20 min incubation at 4 °C under continuous rotation (13 rpm, Dynal MX-1, Life Technologies, Belgium). Cells were then rinsed three times 10 min in 0.05M sodium cacodylate buffer. Post fixation took place in 1% osmium tetroxide in the same buffer for 1 h at room temperature. Afterwards, the cells were rinsed twice with distilled water and were stepwise dehydrated, using ethanol series of increasing concentrations (15-100% ethanol, 10 min each). The cells were subsequently impregnated with a low-viscosity embedding medium (Spurr's resin (Spurr, 1969)), and polymerisation was performed at 70 °C for 8 h. Samples were sectioned on a Reichert Ultracut S (Leica, Vienna, Austria), first semi-thin (0.5 µm) until the region of interest was reached, after which ultra-thin (70 nm) sections were made. Semi-thin sections were studied using a Wild light microscope (Heerbrugg, Switserland). Ultra-thin sections were studied with a Jeol JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60 kV, and pictures were digitized using a Ditabis system (Pforzheim, Germany).

3.8 Statistical analysis

All assays (persistence, supernatant, and gentamycin protection) were performed in triplicate, except for the persistence assay with MOI 1:1. Statistical data analysis was performed on the quantitative data using the software Stata 11.0 (Stata Corporation, College Station, Texas USA). A probability of $p<0.05$ was required for statistical significance. Bacterial counts obtained by the persistence assays were $\log_{10}$ transformed and analyzed for each time point using generalized least squares regressions, with strains as random-effects. Coculture and monoculture counts were compared for every combination of temperature and nutrient condition. To determine strain and/or temperature differences in the absolute number of living amoeba/bacteria (supernatant test) and of intraprotozoan *Yersinia* (gentamycin protection assays), negative binomial regressions were used, including strains as random effects where necessary.

4. Results

4.1 Persistence of *Yersinia* in coculture with *Acanthamoeba castellanii*

In general, for each strain under each experimental condition (temperature and media combination), the survival of *Y. enterocolitica* in both cocultures and controls showed not to be strain-dependent (Fig. 1, small standard errors of the means).

In the cocultures, under nutrient-poor conditions (PAS, Fig. 1A), the number of viable yersiniae did not differ significantly between the different temperatures ($p>0.05$). The number of viable *Yersinia* in both coculture and monoculture conditions at 7 and 25 °C remained almost constant during the 14-day monitoring period, with no significant differences between cocultures and monocultures ($p>0.05$). At 37 °C, the number of viable *Yersinia* in the monocultures decreased earlier than the ones in the cocultures and by day 14 the number of viable *Yersinia* in the monoculture was significant lower than in the cocultures ($p<0.001$).

Under nutrient-rich conditions (PYG, Fig. 1B), the number of viable yersiniae was significantly higher in cocultures at 7 and 25°C compared to 37 °C after 1 day, and significantly higher at 7 °C compared to 25 °C after 6 days ($p<0.001$). However, similar differences between temperatures were obtained in the *Yersinia* monocultures. For each temperature, an initial bacterial growth of 0.5 to 1.5 log cfu/mL was observed, both in cocultures and controls (Fig. 1B). From day 3 onwards, a *status quo* of viable *Yersinia* was observed in both co- and monocultures at 7 °C (*c*. 9.5 log cfu/mL) and in the cocultures at 25 °C (*c*. 8.5 log cfu/mL), whereas the bacterial viability in the monocultures at 25 °C decreased. At 37 °C, no viable *Yersinia* could be recovered after day 3, both in the co- and monocultures. At 7 and 37 °C, no significant difference in viable *Yersinia* counts was detected between the cocultures and the monocultures, whereas at 25 °C the number of viable *Yersinia* in cocultures was significantly higher than in the monocultures from day 6 onwards ($p<0.05$).

For each condition (*i.e*. specific strain, temperature and media combination), light microscopy revealed no visual difference in amoebal cell integrity and density between cocultures and amoebal monocultures during the 14-day monitoring period. In most conditions, the amoebae stayed adherent and pseudopodia and vacuoles were visible, both

in co- and monocultures. By contrast, in PAS-medium at 7 and 25 °C a gradual transformation of trophozoites into resting cyst was observed after 3 and 6 days respectively, in both coculture and monoculture conditions. However, after 14 days, living trophozoites could still be detected. At 37 °C, amoebal viability declined and cyst formation and the presence of amoebal cell debris was observed from day 3-4 onwards in both coculture and amoeba monoculture conditions. Furthermore, from day 6 onwards, no trophozoites could be detected at 37 °C.

To increase the amoebal grazing pressure on *Yersinia*, persistence assays with an MOI 1:1 were also performed at 7, 25 and 37 °C under nutrient-poor conditions, which resulted in similar observations as reported above for MOI 100:1 (data not shown).

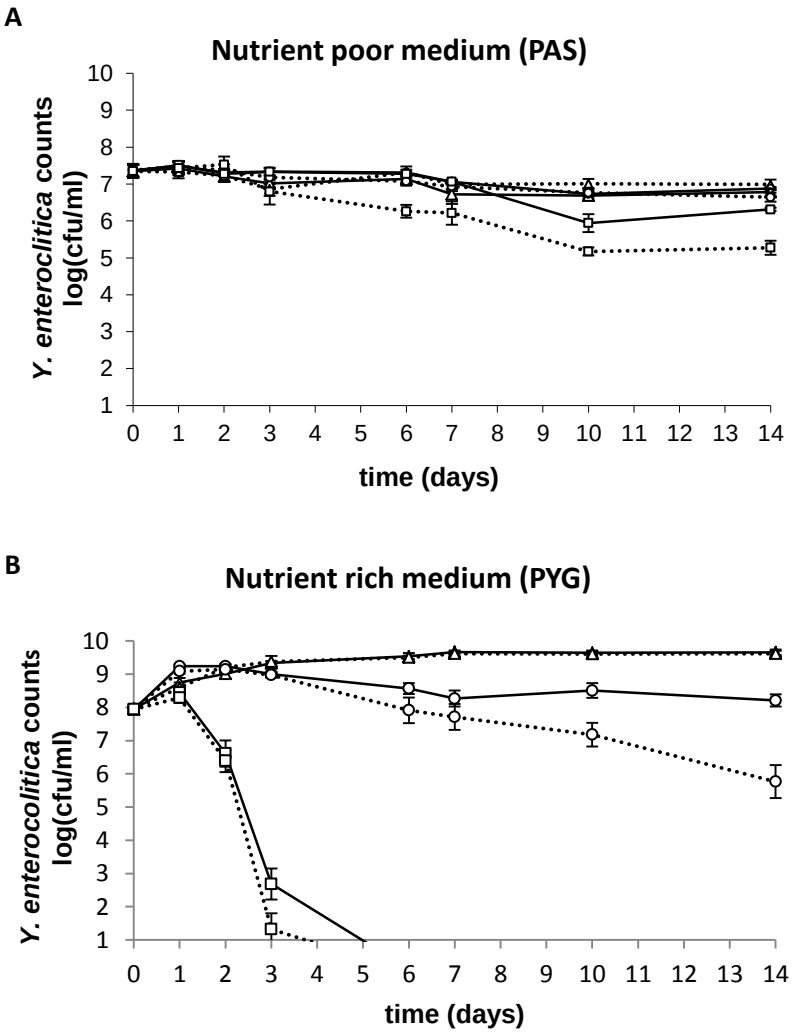


Figure 1. Persistence of *Y. enterocolitica* in coculture with *A. castellanii*
Yersinia enterocolitica were cultivated with (full line) or without *A. castellanii* (dotted line) in nutrient-poor PAS (A) or nutrient-rich PYG medium (B) at different temperatures (Δ 7 °C, o 25 °C, □ 37 °C). MOI 100:1. Values represent the overall means of the four *Y. enterocolitica* strains of the three replicate experiments ± standard error of the mean.

4.2 Effect of released factors on microbial survival and growth

In general, the survival and growth of yersiniae inoculated in amoebal supernatant were similar as seen in the controls. For both, an initial growth of *c*. 1.0 -1.5 log cfu/mL was observed at each temperature. At 7 and 25°C a slight increase *c.* 1 log cfu/mL was detected after time, while at 37 °C, a rapid decrease *c.* 6-8 log cfu/mL of yersiniae viability was observed after 1 day (data not shown).

No differences in amoebal survival and growth between those cultivated in bacterial supernatant and in control medium were observed, except for amoebae inoculated in the supernatant of *Yersinia* strain YeH3- (Fig. 2). Here, significantly lower absolute numbers of viable amoebae were detected in bacterial supernatant than in the control after 3 h incubation at 37 °C ($p<0.001$), after 2 days at 25 °C ($p<0.05$) and after 3 days at 7 °C ($p<0.001$). From 3 h onwards, the number of living amoebae in the supernatant treatment condition at 37 °C was significantly lower compared to the treatment conditions at 25 °C and 7 °C ($p<0.01$). The total number of amoebae, *i.e.* the sum of permeabilized and viable amoebae, remained the same over time (*c.* $5x10^5$ cells/mL).

The initial pH of the TSB-medium before inoculation was 7.2 ± 0.1 units, but it decreased to 5.2 units when YeH3- was cultivated in TSB. The pH-lowering effect of the supernatant of the other strains was less pronounced (5.6-6.5 pH units). Acidified TSB-medium alone (HCl, 5.2 pH units) also caused permeabilization at 7 and 37°C, but to a much lesser extent (*i.e.* maximum 28% permeabilized cells after d4) than the amoeba cultivated in YeH3- supernatant (data not shown).

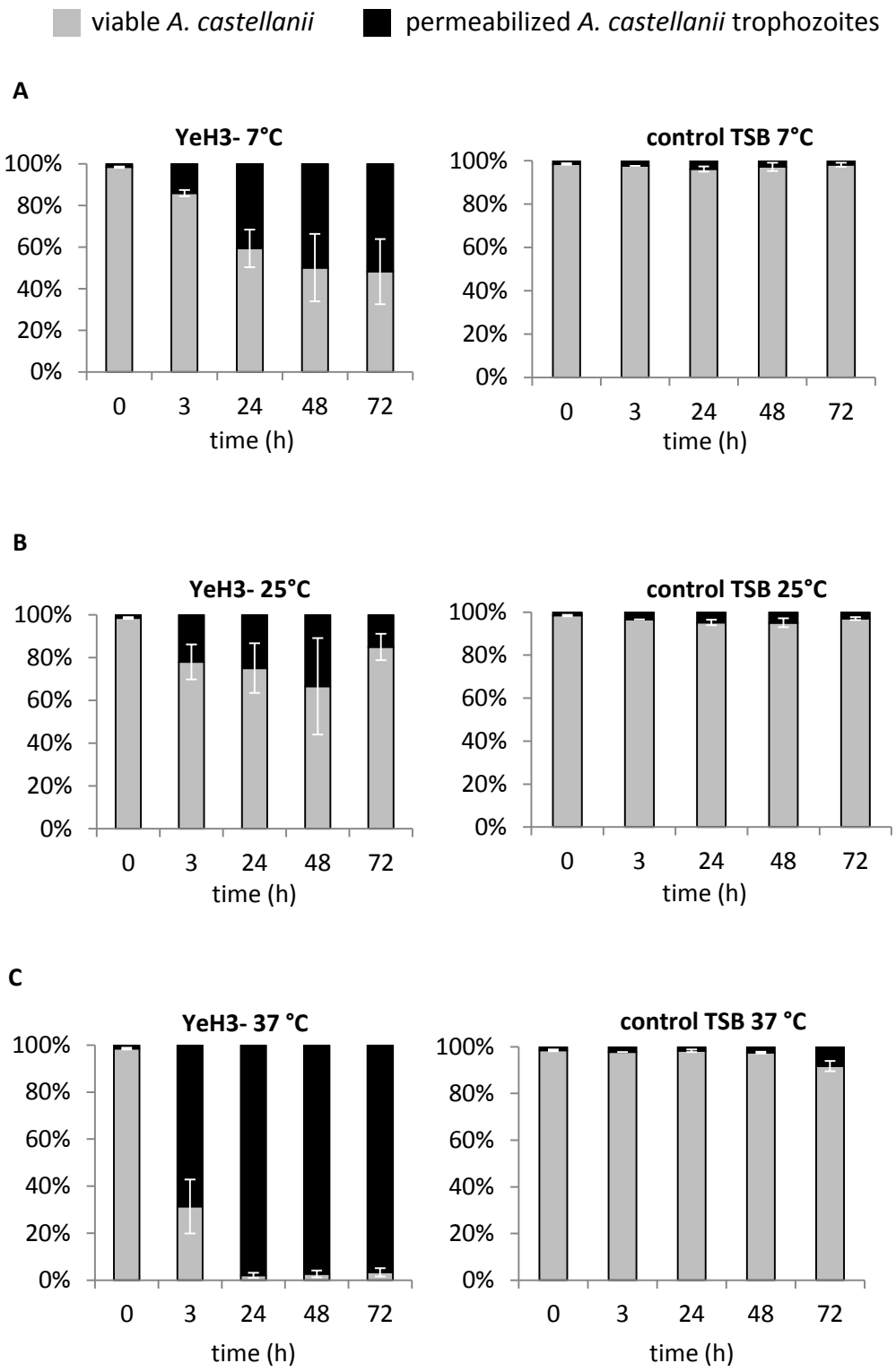


Figure 2. Effect of supernatant from *Y. enterocolitica* strain YeH3- on *A. castellanii*
Percentage of living (indicated in grey) and permeabilized (in black) amoebae related to the total amoebal count, when cultivated in the cell-free supernatant of a *Y. enterocolitica* culture (strain YeH3-) and incubated at 7 °C (A), 25 °C (B) and 37 °C (C). Amoebae were cultivated in the growth medium of the *Yersinia* culture (TSB) as control. Bars represent the mean of three replicate experiments ± standard error of the mean.

4.3 Intraprotozoan survival of *Y. enterocolitica*

For each experimental condition, amoebal counts and cell integrity remained constant over time, in both cocultures and amoeba monoculture. In nutrient-poor medium (PAS) at 25 and 37 °C, no viable intracellular *Yersinia* could be recovered after one day. At 7 °C, no extracellular *Yersinia* were recovered at the 0 h time point, after treatment with 100 µg/mL gentamycin. However, recovery of extracellular *Yersinia* after one day, probably due to the failure of the gentamycin maintenance solution (20 µg/mL) in killing extracellular bacteria in PAS at low temperature, compromised the exact determination of the amount of intracellular *Yersinia*.

In nutrient-rich medium (PYG), *Y. enterocolitica* could survive inside *A. castellanii* (Fig. 3). Over time, all *Y. enterocolitica* strains survived intracellularly for at least 4 days at 7 °C, with 0.71 to 2.8 log cfu/mL detected inside the amoebae. At 25 and 37 °C, the number of intracellular *Yersinia* decreased, with low or no bacterial recovery at the end of the experiment. At each time point, the number of intracellular *Y. enterocolitica* in coculture with *Acanthamoeba* was significantly higher at 7 °C than at 25 °C ($p<0.001$) and 37 °C ($p\leq0.001$).

Although the initial inoculation concentration was the same for each experimental condition (strain and temperature combination), counts were already significantly different between temperature treatments at time point 0 (*i.e*. after a 2 h feeding period followed by a 1 h gentamycin treatment). Strains YeH3+ and YeM3+ had similar concentrations at 0h, whereas the number of viable yersiniae of strain YeH3- was almost 1 log cfu/mL lower. Replicate-counts at the 0 h time point of strain YeH9- at 7 °C varied from 0.53 to 4.50 log cfu/mL (although similar inoculation levels at the start of the experiment), whereas replicates of all other strain/temperature combinations varied much less.

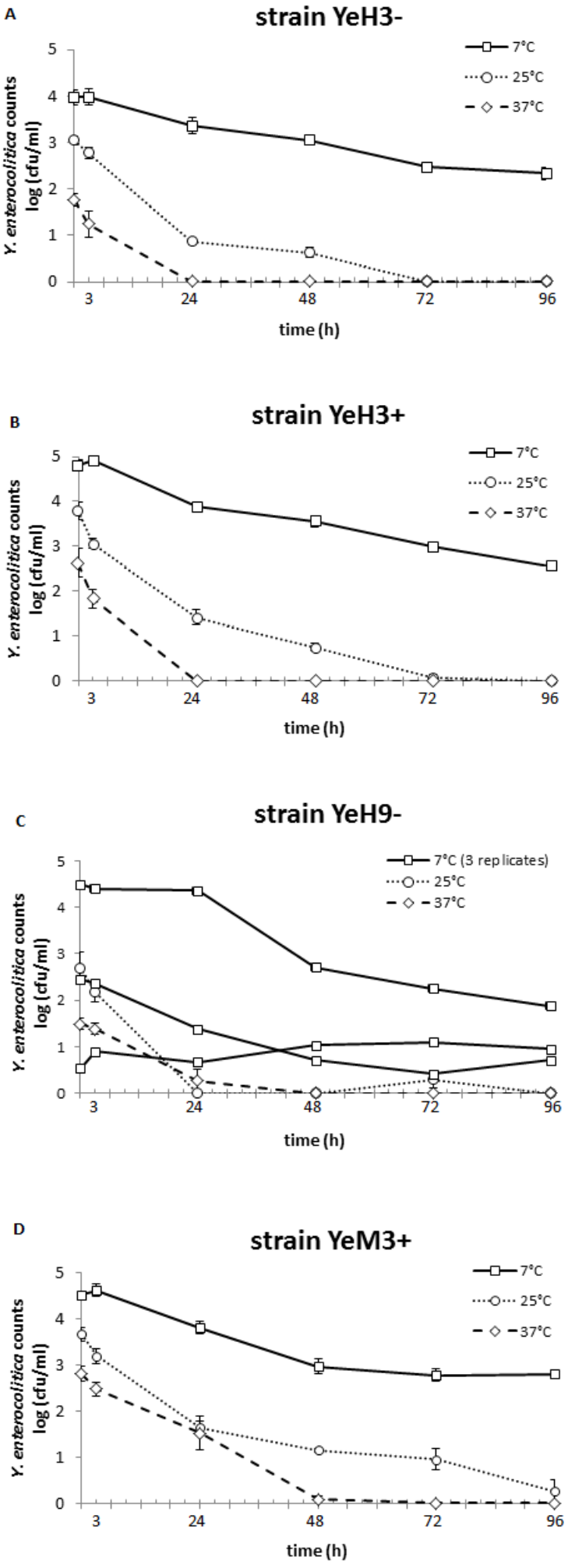


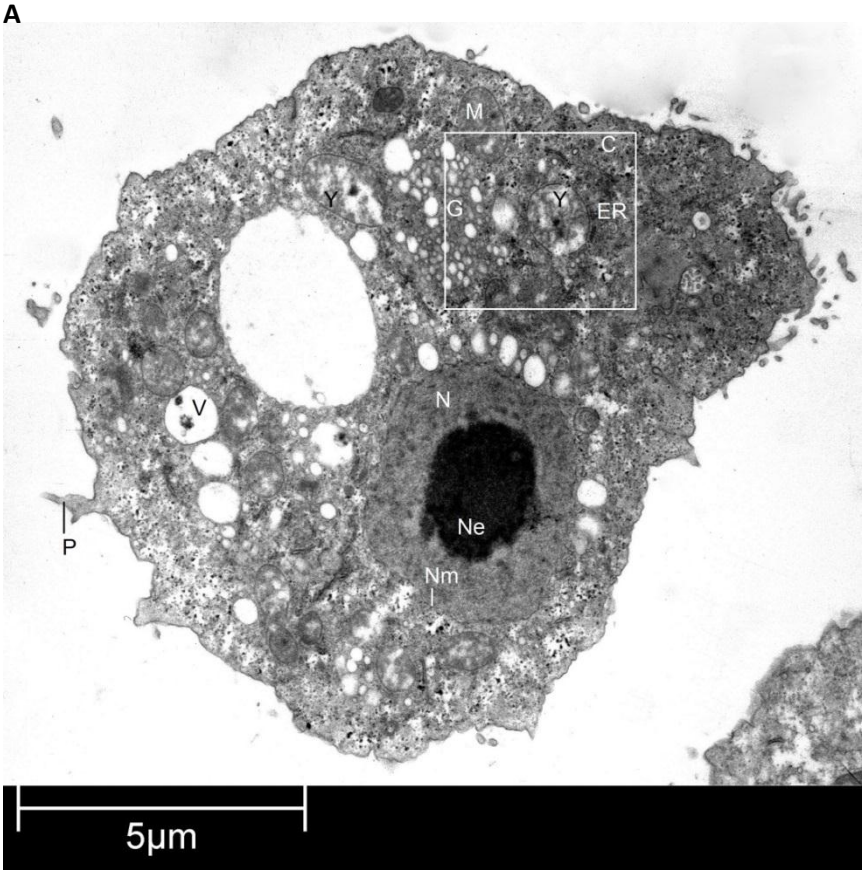
Figure 3. Intracellular viable counts of *Y. enterocolitica* at different incubation temperatures

Acanthamoeba castellanii and *Y. enterocolitica* strain YeH3- (A), YeH3+ (B), YeH9- (C) and YeM3+ (D) were cocultivated in nutrient-rich medium and incubated at different temperatures. Values represent the means of three replicate experiments ± standard error of the mean.

4.4 Localization of intraprotozoan *Y. enterocolitica.*

At each time point, transmission electron microscopy was performed to determine the intra-amoebal location of the bacteria. Pseudopodia and food vacuoles were visible, indicating that the experimental coculture conditions favored normal amoebic activity. After 3 days of cocultivation, the intraprotozoan bacteria were located in the cytoplasm of the amoeba, but not visibly surrounded by an amoebal vacuole membrane (Fig. 4). Host endoplasmic reticulum (ER) was located close to the intracellular bacterium.

A



B

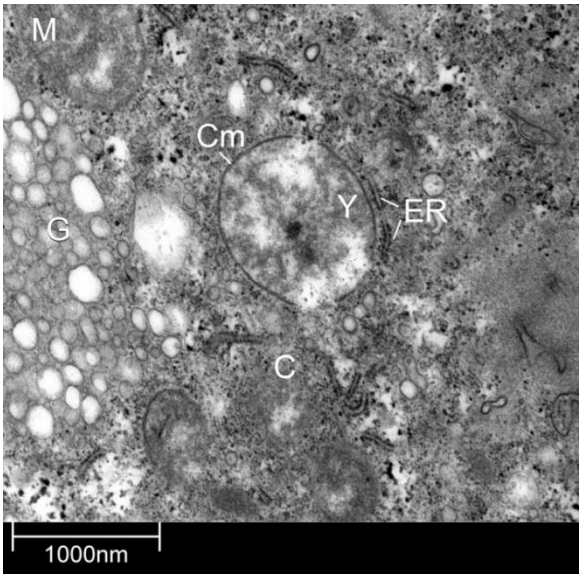


Figure 4. *Acanthamoeba castellanii* trophozoite with internalized *Yersinia enterocolitica*
TEM micrographs of *A. castellanii* incubated for 3 days with *Y. enterocolitica* strain YeH3- in nutrient- rich medium at 7°C **(A)**
(B) P: pseudopodium, V: food vacuole, C: cytoplasm, Nm: nucleus membrane, N: nucleus, Ne: nucleolus, M: mitochondrion, Y: *Yersinia enterocolitica*, ER: endoplasmic reticulum, G: globules, Cm: bacterial cell membrane.

5. Discussion

The present study showed that *Acanthamoeba castellanii* enhances *Yersinia enterocolitica* survival under certain environmental conditions. As *Yersinia* and *Acanthamoeba* share similar ecological and anthropogenic niches, this interaction identifies a potential role of free-living protozoa in the ecology and epidemiology of *Y. enterocolitica*. Though *Acanthamoeba castellanii* is a bacterivorous free-living species that actively grazes on bacteria, with an estimated ingestion rate up to 700 bacteria per amoeba per hour (Corsaro et al., 2013), in the persistence assays, no decreased bacterial viability was observed in the cocultures with *A. castellanii* during the 14-day monitoring period. This proves that *Y. enterocolitica* could survive in presence of *A. castellanii*, although no distinction could be made if the recovered bacteria were internalized in the amoebae (*i.e.* resistant to amoebal digestion) or if it concerned adherent or free-living extracellular bacteria (*i.e.* resistant to amoebal uptake).

Moreover, the persistence assays show that interaction with *A. castellanii* enhances the survival of *Y. enterocolitica* under nutrient-rich conditions at 25 °C (+ 2.4 Log_{10} after 14d) and under nutrient-poor conditions at 37 °C (+ 1 Log_{10} after 14d). However, the presence of cell debris in both cocultures and amoebal monocultures at the latter temperature indicates amoebal lysis, presumably due to nutrient depletion and supra-optimal temperature. A similar decrease in amoebal viability under co- and monoculture conditions has also been reported by Baré *et al.* and Greub *et al.* (Greub et al., 2003; Baré et al., 2010). The formed amoebal cell debris may in turn be used by *Yersinia* as a nutrient source*,* explaining the better survival in the cocultures at 37 °C than in the monocultures. The fact that, after 3 days incubation in nutrient-rich medium at 37 °C, no *Yersinia* could be detected in both cocultures and monocultures, is presumably due to the high metabolic activity of *Yersinia* under these conditions, which led to rapid nutrient depletion.

The present study also revealed that low temperatures and high nutrient availability favor intraprotozoan survival. These conditions are met in industrial food processing settings, at home as well as in natural environments. Although *Yersinia* is a psychrotrophic[4] bacterium

[4] cold-tolerant bacteria that have the ability to grow at low temperatures

and can withstand low temperatures without amoebal protection ((Goverde et al., 1998), *cf*. bacterial monoculture controls in persistence assays), the association can enhance *Y. enterocolitica* survival by physically protecting against chlorine treatment. Chlorine is a commonly used compound to disinfect water and food processing equipment, and increased resistance against chlorine of intra-protozoan *Y. enterocolitica* has been reported (King et al., 1988).

In general, higher numbers of intracellular bacteria were recovered at lower temperatures and for a longer period of time. This is in parallel with a recently published study where a *Yersinia* strain was not recovered from *Acanthamoeba polyphaga* after 2 days at 30 °C (Anacarso et al., 2011). The high intraprotozoan survival observed at 7 °C could be due to slower amoebal phagocytic digestion (Khan, 2009), bacterial circumvention of amoebal digestion by escaping into the amoebal cytosol, as observed by TEM, or both. Further research is needed to elucidate *Yersinia*'s intracellular survival mechanisms.

The number of viable intracellular *Yersinia* decreased with time. This could be due to (partial) bacterial digestion, bacterial release in the environment and subsequent bacterial killing by gentamycin or to a switch to a viable but non cultivable state (VBNC) of the intracellular *Yersinia* (Smith et al., 1994). Partial intraprotozoan digestion has already been described for other bacterial pathogens (Gaze et al., 2003; Tezcan-Merdol et al., 2004; Pickup et al., 2007; Baré et al., 2010). Linking back with the results of the persistence assay, where no reduction in viable bacteria was observed under most cocultivation conditions, another explanation could be that intraprotozoan *Yersinia* are released or trigger their release in the extra-amoebal environment (Anacarso et al., 2011). In the gentamycin protection assay these extracellular bacteria are killed by the gentamycin maintenance concentration, whereas extracellular growth/survival is possible for yersiniae in the persistence assay. In the gentamycin protection assay, no amoebal growth was observed, which confirmed that the amoebae did not appear to obtain adequate nutrients from *Yersinia* to increase their concentration.

Strain variation in intracellular survival capacity was detected from the start of the experiments onwards, which could be due to variation in the ability of the strains to invade or to become internalized by *Acanthamoeba* or to their intrinsic intracellular survival

capacities. This study indicates that the presence of the *Yersinia* virulence plasmid favors bacterial uptake and/or intracellular survival, as at time point zero, the number of intracellular yersiniae was higher for the plasmid carrying YeH3+ strain than for its plasmid cured derivate (YeH3-).

For the first time, TEM was used to visualize the exact subcellular location of *Yersinia* inside *A. castellanii*. Images show that the intracellular yersiniae were located in the cytosol and were not visibly surrounded by an amoebal vacuole membrane. This may indicate that *Y. enterocolitica* is able to circumvent the normal digestion pathway by escaping from the food vacuole to the cytosol. This post-ingestional adaptation mechanism to avoid protozoan digestion has also been described for members of the genus *Rickettsia* (Whitworth et al., 2005). In addition, the *Y. enterocolitica* were located close to the host endoplasmic reticulum (ER), which may be advantageous as the bacteria have easy access to newly synthesized host proteins. Further research is necessary to reveal if the co-localization of host ER and intracellular yersiniae is coincidental or if there exists a *Yersinia* mechanism to repose the host ER, as described for *Legionella pneumophila* (Robinson and Roy, 2006; de Felipe et al., 2008).

In the supernatant assays, the supernatant of strain YeH3- (4/O:3, pYV-) had a temperature-dependent permeabilizing effect on the amoebae. It is not clear if this reflects a direct or indirect effect on the amoebal cell membrane integrity. During bacterial cultivation at the start of the experiments, the pH of this supernatant had already decreased compared to the supernatant of the other strains. However, the permeabilizing effect could not be explained by a pH reduction as such. As this permeabilizing effect was not observed in the persistence assays, a hypothesis could be that direct cell contact between amoebae and bacteria may activate a host defense mechanism to prevent permeabilization. Alternatively, the effect can be concentration-dependent, as washing steps in the persistence assay protocol could have lowered the amount of permeabilizing factors. Further research is needed to identify the cause of this permeabilization (influenced by temperature) and the observed pH drop, with attention to the production of a potential bacterial toxin or a metabolite with anti-protozoan activity (Matz and Kjelleberg, 2005).

The association of *Y. enterocolitica* with protozoa may have relevant ecological and epidemiological implications. Besides enhancing *Y. enterocolitica* survival in their presence, being intra- or extracellular, free-living protozoa have the potential to act as a reservoir, vector, infection route, biological gym and evolutionary crib for intracellular *Yersinia enterocolitica,* as previously described for other pathogenic bacteria (Cirillo et al., 1997; Greub and Raoult, 2004; Thomas et al., 2010; Siddiqui and Khan, 2012).

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CHAPTER IV

Transmission electron microscopy sample preparation protocols for the ultrastructural study of cysts of free-living protozoa

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1. Abstract

Cysts of free-living protozoa may have an impact on the ecology and epidemiology of (pathogenic) bacteria, as they may act as a vector and shelter against hash environmental conditions. Detection and localization of intracystic bacteria and examination of the en- and excystment dynamics is a major challenge, as no detailed protocols for ultrastructural analysis of cysts are available yet. Transmission Electron Microscopy (TEM) is ideally suited for those analysis, however, conventional TEM protocols are not satisfactory for cysts of free-living protozoa.

In the present paper, four protocols for TEM sample preparation of cysts were designed and tested. Two protocols, one based on chemical fixation in coated well plates and one on High Pressure freezing, were selected as the most effective protocols for TEM-based ultrastructural studies of cysts. The proposed protocols will allow a better analysis of the cysts structures and a better understanding of bacterial survival mechanisms in cysts.

2. Introduction

Free-living protozoa are eukaryotic microorganisms which are common inhabitants of natural and anthropogenic aquatic, terrestrial and aerial environments (Khan, 2009; Vaerewijck et al., 2014). They play a critical role in microbial ecosystems as they are major grazers on bacteria and other microbial eukaryotes including other protozoa and microalgae (Matz and Jurgens, 2005; Khan et al., 2014). Formation of cysts is an integral part of the life cycle of many protozoa which enables them to survive harsh physical and chemical environmental conditions (Coulon et al., 2010; Dupuy et al., 2014). It is becoming increasingly clear that free-living protozoa may also play a pivotal role in the ecology and epidemiology of pathogenic bacteria, such as *Legionella* and *Campylobacter* (Snelling et al., 2008; Baré et al., 2010; Price et al., 2014). These bacteria can either resist protozoan uptake, or are internalized but resist digestion and can later be released from the protozoon. As such, the protozoon can function as a reservoir, vector and/or shelter for these pathogens (Vaerewijck et al., 2014). It has recently been shown that some of these pathogens can also survive the protozoon en- and excystment process (Ben Salah and Drancourt, 2010; Lambrecht et al., 2015), which greatly enhances their survival and dispersal potential in natural and anthropogenic environments.

A better understanding of the bacteria-protozoon interaction requires a more detailed knowledge of the exact association mode and bacterial survival mechanisms (e.g.Robinson and Roy, 2006; Denoncourt et al., 2014) especially in the cyst stage. Transmission Electron Microscopy (TEM) is ideally suited for detecting and localizing the bacteria inside protozoa, and to study the dynamics of en- and excystment.

For protozoan trophozoites (*i.e*. the vegetative life stages), well-established TEM protocols are available in literature (Inglis et al., 2000; Smirnov and Brown, 2004). To date however, no satisfactory protocols are available yet for TEM studies of protozoan cysts. In routine TEM-protocols, fixation and subsequent staining and dehydration steps are all performed in centrifugation tubes, which is not optimal for cysts. Often, free-living protozoan cysts have a thick, sometimes layered cell wall and are prone to stick strongly to the walls of plastic centrifugation tubes after fixation, resulting in failure to form a dense pellet. This leads to massive loss of cysts during the numerous centrifugation steps needed to prepare the TEM

sample. The few published TEM-protocols for cysts are often vague and incomplete. Essential technical specifications like centrifugation speed, duration, incubation temperatures, and the kind of recipients used are not always mentioned (e.g.Adekambi et al., 2006; Ben Salah and Drancourt, 2010). This turns TEM sample preparation of cysts into a trial-and-error process, which is very time-consuming and expensive.

In the present paper, we developed three chemical fixation protocols, build on existing sample preparation procedures (Hayat, 2000; Bert et al., 2003; Claeys et al., 2004; Smirnov and Brown, 2004). In addition, for the first time, a HPF-AFS protocol for TEM studies of *Acanthamoeba* cysts was designed. All protocols were evaluated for their usefulness in obtaining intact, high quality TEM images of *Acanthamoeba* cysts, which allow detection of intracellular structures and (if present) bacteria. *Acanthamoeba* is a well characterized, free-living model amoeba and is frequently used in co-culture studies with bacteria (e.g.Baré et al., 2010; Lambrecht et al., 2013; Yousuf et al., 2013).

3. Materials and Methods

Four sample preparation protocols for TEM analyses of cysts of free-living protozoa were tested:

(**A**) a **chemical fixation protocol** in which all steps are performed in centrifugation tubes, as frequently used for protozoan trophozoites,

(**B**) a **chemical fixation protocol** in which all steps are performed in coated well plates,

(**C**) a **chemical fixation protocol** whereby initial chemical fixation is performed in centrifugation tubes, but subsequent sample preparation steps are performed in coated well plates,

(**D**) a **High Pressure Freezing and Automatic Freeze Substitution (HPF-AFS)** protocol.

All protocols were performed on both xenic and/or axenic cultures of *Acanthamoeba castellanii.* Each protocol consists of the following steps: (1) cultivation, (2) fixation, (3) postfixation and *en bloc* staining (4) dehydration and embedding, (5) ultrathin sectioning and staining (6) and visualization.

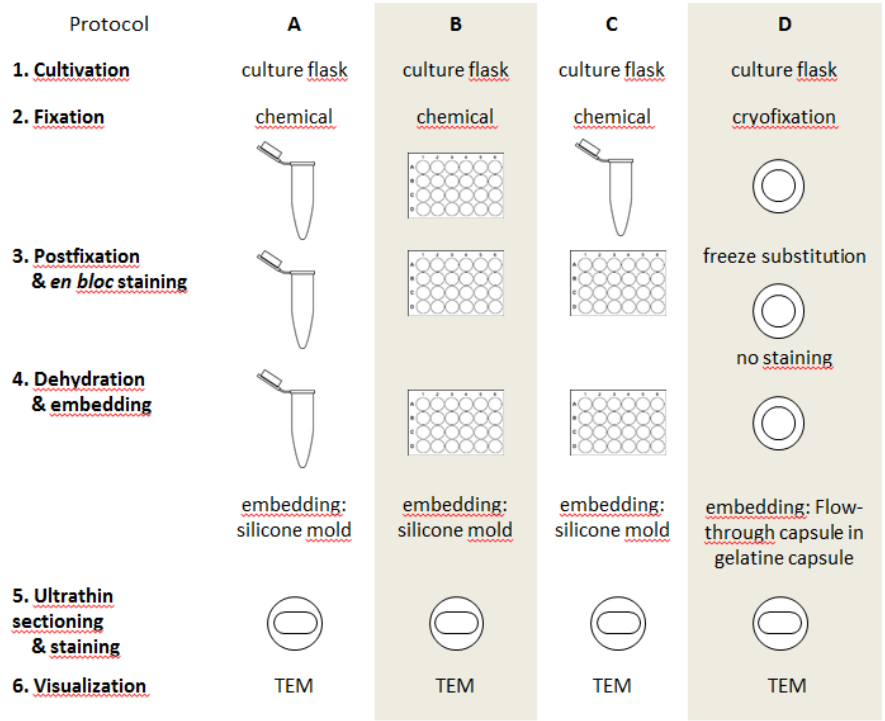


Figure 1: Overview of the four TEM sample preparation techniques for TEM analysis of cysts of free-living protozoa.

3.1. Cultivation of *Acanthamoeba castellanii* and induction of cyst formation

In each TEM sample preparation protocol (**A, B, C, D**), mature cysts (6 days old) of *Acanthamoeba castellanii* were used. *Acanthamoeba castellanii* trophozoites (ATCC30234, American Type Culture Collection) were maintained in Proteose peptone Yeast extract Glucose medium (PYG, ATCC-recipe, http//www.lgcstandards-atcc.org) at 25°C in 75 cm^2 culture flasks (TPP, Trasadingen, Switzerland). Encystment of *A. castellanii* trophozoites was induced by incubation of the amoebae in 10 ml High Saline (HS) buffer at 25°C for 6 days in 25 cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland) as described by Lambrecht (2013). In short, the initial concentration of trophozoites was determined with a Fuchs-Rosenthal counting chamber and adjusted to $5x10^5$ cells/ml HS. A higher concentration increased cyst clustering, which made cysts less prone to adhere to the bottom of the culture flasks, impairing the later steps in the protocol (data not shown). The encystment process was monitored under a light microscope (Olympus CKX41). After 6 days, *c*. 96 % of the cells were encysted and adhered to the bottom of the culture flasks. Remaining trophozoites and extracellular bacteria (in xenic cultures) were removed by 3% HCl (v/v in H_2O) treatment for at least 2 h, followed by a washing step with Page Amoeba Saline (PAS, ATCC recipe, http//www.lgcstandards-atcc.org) and a gentamycin treatment (100 µg/ml HS) for 2 h at 25°C , all in tissue culture flasks.

3.2. Fixation of amoebal cysts

Adherent cysts were washed twice with PAS and resuspended in 5 ml PAS. Cysts were removed from the bottom of the flasks by cell scraping (Cell Scraper TPP, Trasadingen, Switserland), transferred to 1.5 ml polypropylene tubes and centrifuged (425xg, 6 min, 4°C). If the pellets were too small for further analysis, cysts were allowed to sediment in the centrifuge tube for 30 min and were centrifuged once more (425xg, 6 min, 4°C). The supernatant was discarded. From this point onwards, different fixation procedures were applied **(A to D)**.

(A, C) The cysts in the centrifugation tubes were gently dissolved in 1 ml 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, Aurion, Wageningen, The Netherlands) at room temperature for 1 h under rotation (Dynal Mx-1, 8 rpm, Invitrogen, Merelbeke, Belgium).

Cells were then centrifuged (425xg, 6 min, 4°C) and 2/3 of the total supernatant was gently removed and replaced by 1 ml 0.1 M sodium cacodylate buffer. This washing step was repeated twice. Subsequently, pellets were suspended in 1ml 0.1 M sodium cacodylate buffer by gently pipetting up and down . For **protocol A**, cyst suspensions were stored in the centrifugation tubes overnight at 4°C, whereas for **protocol C,** 1 ml of the cyst suspension was pipetted into coated wells (24-well culture plate, TPP, Trasadingen, Switserland). Preliminary results showed that after fixation, cysts exhibited reduced adhesive capacity on the plastic bottom of the well plates. To ensure that cysts stayed attached to the well plates throughout the whole procedure, collagen coated cover glasses were used. This procedure is frequently used to support adherent cell growth of other cell types (De Regge et al., 2010). For the coating, collagen powder (Collagen from Rat tail Type I, Sigma-Alderich, St Louis, US) was dissolved in 0.01 M acetic acid to obtain a 0.25% (w/v) collagen solution. The solution was stirred for 1-1.5 h at 37°C until a viscous suspension was obtained. This stock solution was stored at 4°C. Before use, the stock solution was diluted four times in 60% ethanol. Glass inserts (Ø 13mm, Knittel, Braunschweig, Germany) were put in 24-well plates and 150 µl of the collagen solution was pipetted onto each glass insert and allowed to dry overnight in a laminar flow cabinet at room temperature. Wells with the inserts inside were washed twice with 200 µl sterile H_2O. Afterwards, the 1 ml cyst suspension (*c.* $5x10^5$ cysts/ml) was added to the wells and centrifuged at 42xg for 2 min (Eppendorf 5810R swing bucket rotor with plate bucket) to enhance cyst attachment to the bottom of the coated plates. Plates were sealed with parafilm and put in the refrigerator at 4°C overnight.

(B) In protocol B, the entire fixation process was performed in 24-well culture plates (TPP, Trasadingen, Switserland) with coated inserts. Cysts at a concentration of $5x10^5$ cells/ml were seeded on the coated inserts (1ml/insert) in 24-well plates, centrifuged (42xg, 2 min.) and incubated overnight at 25°C. Adherent cysts on the bottom of the coated wells were treated with 1 ml 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, Aurion, Wageningen, The Netherlands) for 1 h at room temperature. Afterwards, wells were washed trice with 1 ml 0.1 M sodiumcacodylate buffer and stored overnight at 4°C.

(D) Protocol D makes use of cryofixation at high pressure (High Pressure Freezing, HPF) and freeze-subsitution. The cysts suspension was gently mixed (1:1) with 50 µl 40% w/v Bovine Serum Albumin (Sigma-Alderich, , St Louis, US). Copper membrane carriers (Leica, Vienna,

Austria) were treated and covered with 1% egg lecithin (Sigma-Alderich, St Louis, US) and filled with the cyst suspension. Afterwards, cysts were frozen very quickly with liquid nitrogen under 2000 bar pressure (EMPact, Leica, Microsystems, Vienna, Austria).

3.3. Postfixation and *en bloc* staining

(A) The cells that were fixed in centrifugation tubes, were harvested (425xg, 5 min) and washed with 1 ml 0.1M sodium cacodylate buffer. Postfixation was performed with potassium ferricyanide reduced osmiumtetroxide (1% w/v OsO_4 (Aurion, Wageningen, The Netherlands) and 1.6 % $K_3Fe(CN)_6$ w/v in 0.134 M sodium cacodylate buffer (EMS, Hatfield, UK) for 1 h at room temperature. The cells were then rinsed three times 30 min with bi-distilled water. Postfixation was followed by *en bloc* staining for 1 h in 1 ml 1% uranyl acetate (Aurion, Wageningen, The Netherlands) in bi-distilled water. The centrifugation tubes were wrapped in aluminium foil for protection against light. All incubation steps were performed on a rotor (Dynal Mx-1, 8 rpm) to ensure optimal contact between cells and products.

(B,C) For the samples treated in coated well plates, rinsing, post-fixation and *en bloc* staining was performed as described above, except that all steps were performed in the coated well plates. The plates were sealed with parafilm and all incubation steps were performed on a shaker. During *en bloc* staining, plates were wrapped in aluminum foil.

(D) Post-fixation (1% OsO_4, 0.1 % glutaraldehyde) of the cryofixed samples was performed during dehydration (see below). There was no *en bloc* staining.

3.4. Dehydration and embedding

(A) Cysts were harvested (425xg, 5 min) and rinsed three times with bi-distilled water for 30 min on a rotor (Dynal Mx-1, 8 rpm) at room temperature. Afterwards, cysts were dehydrated on a rotor at room temperature over 10 minutes using graded ethanol series from 15%, 30%, 50%, 70%, 90%, 100%. Following dehydration, cysts were embedded in low viscosity embedding medium Spurr resin (Elektron Microscopy Sciences, EMS, Hatfield, UK). First,

cysts were incubated in an absolute ethanol:Spurr (3:1) solution for 1 h, followed by an absolute ethanol:Spurr (1:1) solution for 2 h. Each incubation step took place at room temperature on a rotor. Afterwards, cysts were resuspended in an absolute ethanol:Spurr (1:3) solution for 4 h at 4°C on a rotor, followed by incubation in 100 % Spurr at 4°C overnight on a rotor. Subsequently, the pure Spurr was replaced with fresh pure Spurr (8 h, on a rotor, 4°C), three more times. Resin polymerization was performed in silicone molds at 70°C for 8 h.

(B, C) Adherent cysts in the culture plates were rinsed and gradually dehydrated, as described above, except that all steps were performed in the coated well plates instead of in centrifugation tubes. All incubation steps were performed on a shaker. After graded embedding in ethanol:Spurr (3:1, 1:1, 1:3 and 3x 100% Spurr) as described above, inserts with adherent cysts were gently removed from the wells and placed on top of the slots of a silicone capsule mold (12 cavities, 8 mm Ø, 16 mm high, EMS, Hatfield, UK) filled with 100% Spurr resin, with adherent cysts on the glass insert facing towards the Spurr resin. Resin polymerization was performed by placing the mold at 70°C for 8 h. The glass insert was gently removed, with the remaining cysts attached to the resin.

(D) After cryo-fixation, cells were freeze-substituted with dry acetone in combination with 1% OsO_4, 0.1 % glutaraldehyde and 2% H_2O (v/v). Freeze substitution was carried out in the automated Leica EM AFS unit (Leica, Vienna, Austria) as follows: -90°C for 27 h, 2°C/h increase for 15 h, -60°C for 12 h, 2°C/h increase for 15 h, -30°C for 32 h and 2°C/h increase for 17 h. Carriers were rinsed three times 1 h with dry acetone at 4°C and impregnated in Spurr as described in 4 (A), but with aceton instead of ethanol. Polymerization took place at 70°C for 8 h in flow-through capsules in gelatin capsules (Leica, Microsystems, Vienna, Austria).

3.5. Ultrathin sectioning and staining

(A, B, C, D) Ultrathin sections (80 nm) were cut using a Leica Ultracut S ultramicrotome (Leica, Vienna, Austria) with diamond knife (DiATOME Ltd., Biel, Switzerland) and collected on formvar-coated (EMS, Hatfield, UK) copper single slot grids (2x1 mm, Agar Scientific, Stansed, UK). Sections were ultra-stained in a Leica EM AC20 (Leica, Vienna, Austria) for 30 min in 0.5% uranyl acetate at 20°C and for 7 min in 3% lead citrate at 20°C.

3.6. Visualization

(A, B, C, D) Sections were studied with a Jeol JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60 kV, and pictures were digitized using a Ditabis system (Pforzheim, Germany).

4. Results and discussion

Cysts of free-living protozoa receive more and more attention as they can act as a shelter and vector for internalized bacteria and hence may play a role in the ecology and epidemiology of bacteria. Transmission Electron Microscopy (TEM) represents an important tool for detection and studying ultracellular structures, however, the currently published protocols are not satisfactory/suitable for protozoan cyst (El-Etr et al., 2009; Abd et al., 2010; Abd et al., 2011). As such, it was necessary to assess different sample preparation procedures and propose (an) optimized protocol(s) that enables effective sample preparation of free-living protozoan- cysts for TEM-images.

In the present study, four cyst fixation techniques for TEM sample preparation were tested (Figure 1 & Table 1). The chemical fixation protocol in which all steps are performed in centrifugation tubes **(A)** resulted in loss of cysts due to their sticking to the wall of the centrifugation tubes as described before. Few or no cysts were left for embedding. To eliminate these yield-reducing-centrifugation steps, we tried to chemically fix adherent cysts in well plates on collagen coated inserts **(B)**. Although initially the cysts strongly adhered to the coated inserts, cysts were floating soon after fixation. After subsequent washing steps in buffer, most of the cysts were lost, although the few remaining cysts adhered again to the coated well plates. These findings led us to use an new, optimized chemical fixation protocol **(C)**, whereby initial chemical fixation is performed in centrifugation tubes and subsequent sample preparation steps (postfixation up to embedding) are performed in coated well plates. To our knowledge this is the first study whereby a chemical fixation protocol is used which makes use of collagen-coated glass inserts to promote cysts adhesion and in which TEM cyst samples are prepared in well plates.

High Pressure Freezing and Automatic Freeze-Substitution (**D**) is an alternative to chemical fixation. Here we describe for the first time that HPF-AFS can also successfully be used for TEM sample preparation for cysts of free-living protozoa.

This study demonstrates that both the optimized chemical protocol for cysts sample preparation (**C**) and the cryofixation protocol (**D**) results in detailed ultrastructural images of cysts (Figure 2). Cellular structures were conserved as demonstrated by an intact double-layered cyst wall with a clear peptidoglycan layer, nucleus, mitochondria and, if present,

intact intracellular bacteria. Both protocols are easy to perform and have a higher cyst yield compared to the chemical fixation protocol frequently used for protozoan trophozoites **(A)** in which all steps are performed in centrifugation tubes.

Although the cryofixation protocol has several advantages including automatization, less chance of artefacts and preservation of delicate, fine, and/or labile intracellular fine structures (eg microtubuli), it has some limitations (complete overview in Table 2). When performing experiments at well–defined time points, HPF-AFS is not the best option as the samples need to be put in the HPF-AFS device at the specified time point. This poses problems for laboratories without in-house HPF-AFS facilities. Moreover, HPF takes 24 h and an AFS run takes 118 h, therefore, multiple devices are needed when sampling is performed with short time-intervals. In contrast, chemical fixation can be easily performed in a basic laboratory by adding 2.5% glutaraldehyde to the samples at the desired monitoring time point.

Although tested on cysts of the free-living model protozoon *A. castellanii*, we suggest that the TEM-protocols can also be used for other cysts, weakly adherent cells and fragile cells (without the need for multiple centrifugation steps), although the protocols need to be further validated.

In conclusion, the present proposed protocols (**C, D**) make it possible to obtain high quality TEM images of cysts, which will be highly relevant for (i) ultrastructure analyses of cysts itself and (ii) infection assays, as it will allow a better understanding of bacterial survival mechanisms and conditions in free-living protozoa.

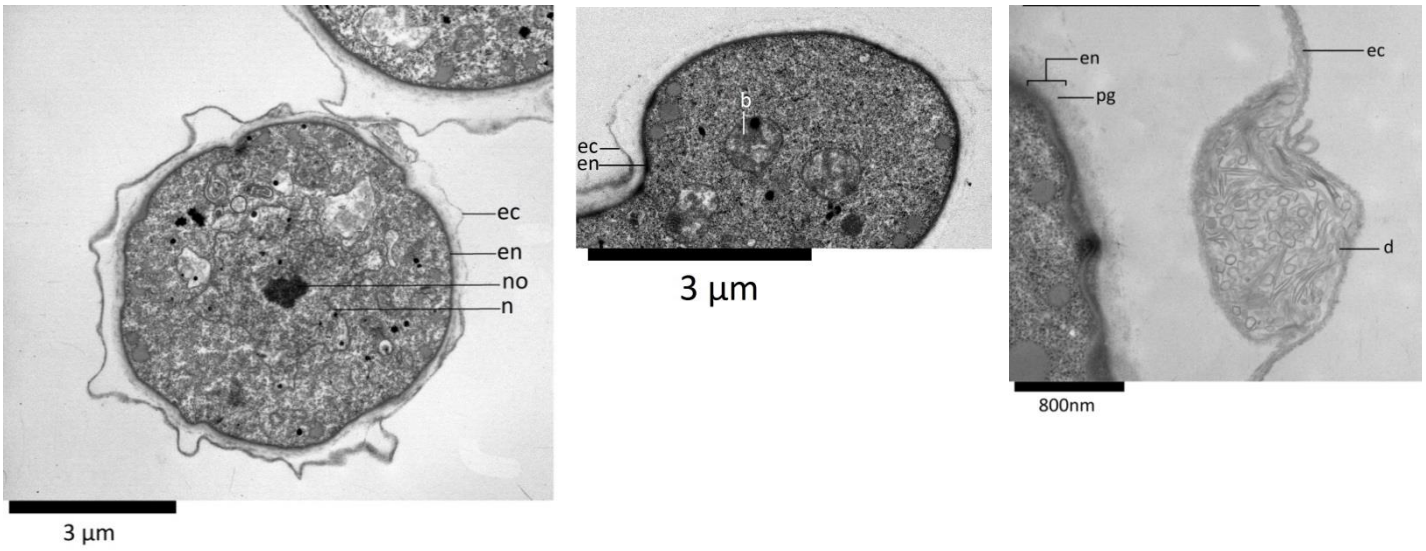
Table 1: Overview of the different cyst fixation techniques

Protocol	Evaluation/main drawbacks	Outcome
A: chemical fixation of suspended cysts in centrifugation tubes	No dense pellet after several centrifugation steps; cysts stick to walls tube	X
B: chemical fixation of adherent cysts in coated well plates	No cysts adherence after fixation, loss of cysts in subsequent steps	X
C: chemical fixation whereby initial fixation is performed in centrifugation tubes and subsequent steps in coated well plates	Very good fixation, cysts stay attached during TEM protocol	V
D: High Pressure Freezing Automatic and Freeze Substitution	Very good fixation	V
X: negative outcome, V: positive outcome, protocol is recommended for fixation of cysts		

Table 2: Comparison of two successful cyst fixation techniques

Features	Protocol C: chemical fixation, combination centrifugation tubes and well plates	Protocol D: High Pressure Freezing and Freeze Substitution
Time-investment	Labour-intensive, hands-on, time-consuming	Fixation: labour-intensive, training required, AFS: automated system
Sample numbers	Easily > 40 samples/day, depends on how many samples one person can handle a day.	20 samples within one run (approx. 1week)
Lab infrastructure	Basic laboratory equipment needed (centrifuge, pipet, fume hood)	Expensive equipment needed (EMPACT cryopreservation device, Automatic Freeze substitution device)
Represents true intracellular structure	Cells can undergo structural changes between first contact with fixative and completion of fixation	Fixation within milliseconds, simultaneous immobilization of all macromolecular components. Preserves delicate, fine, and/or labile intracellular fine structures (e.g. microtubuli). Less chance of artefacts
Sampling plan	Easy to monitor multiple samples at different, well- defined time-points. Short time between sampling and fixation as fixation only requires addition of 2.5% glutaraldehyde	Experiments with different time-points can pose problems, as samples must be put immediately in a HPF-AFS device (one run = approx.. 1 week). Multiple devices needed when working with short time-intervals.

A



B

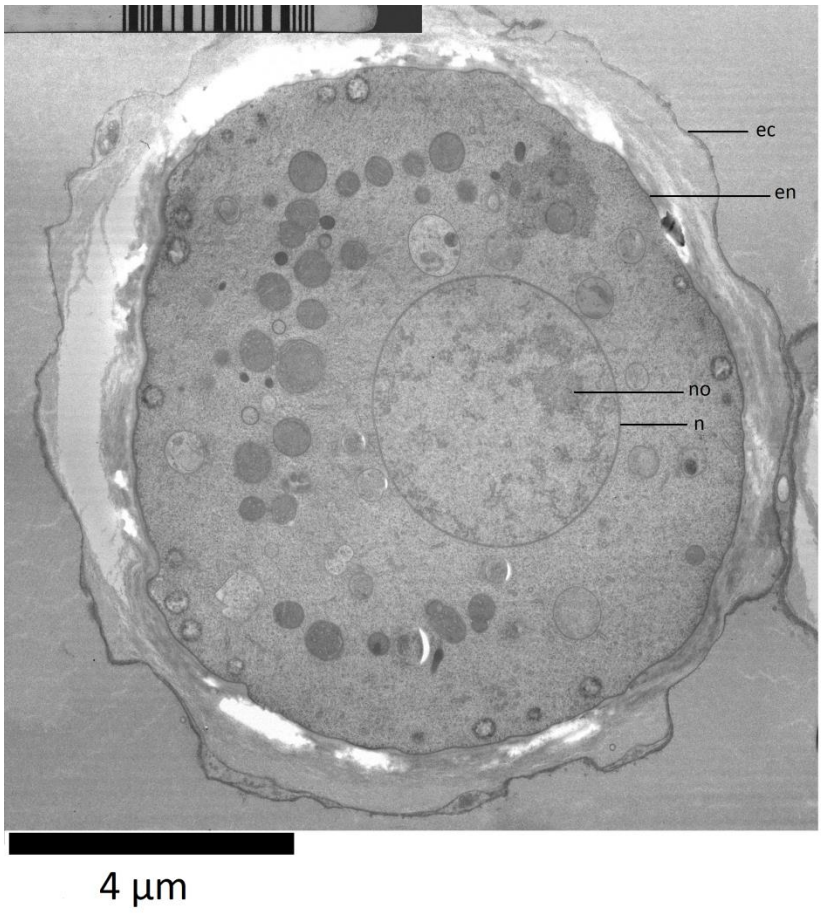


Figure 2: Transmission electron microscopy images of *A. castellanii* cysts
(A) Chemical fixation (protocol C), (B) High Pressure Freezing and Automatic Freeze Substitution (protocol D)
b: bacterium, d: cell wall deposits, ec: ectocyst, en: endocyst, pg: peptidoglycan layer, n: nucleus, no: nucleolus

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CHAPTER V

Protozoan cysts: a potential survival niche and protective shelter for foodborne pathogenic bacteria

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1. Abstract

The production of cysts, an integral part of the life cycle of many free-living protozoa, allows these organisms to survive adverse environmental conditions. Given the prevalence of free-living protozoa in food-related environments, it is hypothesized that these organisms play an important, yet currently underinvestigated role in the epidemiology of foodborne pathogenic bacteria. Intracystic bacterial survival is highly relevant, as this would allow bacteria to survive the stringent cleaning and disinfection measures applied in food-related environments. The present study shows that strains of widespread and important foodborne bacteria (*Salmonella enterica*, *Escherichia coli, Yersinia enterocolitica*, and *Listeria monocytogenes)* survive inside cysts of the ubiquitous amoeba *Acanthamoeba castellanii,* even when exposed to either antibiotic treatment (100 µg/ml gentamycin) or to highly acidic conditions (pH 0.2) and resume active growth in broth media following excystment.

While species-specific differences in trophozoite invasion/uptake efficiency were observed, these could not be correlated with the pronounced differences in intracystic survival period, ranging from d0 for *E. coli O:157* up to 21d for both *S. enterica* strains.

The viability and the encystation process of the amoebae was not markedly affected by bacteria, except for strain *Y. enterocolitica* 2/O:9. For this strain a significant delay in encystment was observed.

Up to 53% of the cysts were infected with pathogenic bacteria, which were located in the cyst cytosol. Our study suggests that the role of free-living protozoa and especially their cysts in the persistence and epidemiology of foodborne bacterial pathogens in food-related environments may be much more important than hitherto assumed.

2. Introduction

Foodborne pathogenic bacteria are a major cause of foodborne illness and have important implications for human public health along with economic consequences (Scharff, 2012). Despite thorough disinfection protocols and hygiene monitoring during food production and processing, pathogenic bacteria often persist in food-related environments and on food, suggesting that our knowledge about the transmission routes and epidemiology of foodborne pathogenic bacteria is still incomplete.

Recent studies have shown that bacteria can benefit from intracellular associations with free-living protozoa (FLP) (Matz and Kjelleberg, 2005; Vaerewijck et al., 2014), heterotrophic eukaryotic microorganisms which are common in natural aquatic and terrestrial ecosystems (Hausmann et al., 2003). Although FLP feed on bacteria, some bacteria resist digestion. These so-called digestion-resistant bacteria can survive and even grow inside their FLP hosts (Anacarso et al., 2011). These hosts may effectively act as a reservoir, shelter and vector for the bacteria, and can as such play an important role in their ecology (Greub and Raoult, 2004; Vaerewijck et al., 2014). Intracellular association with FLP has also been demonstrated for human pathogenic bacteria (Miltner and Bermudez, 2000; Jules and Buchrieser, 2007), including food-related pathogens (e.g.Baré et al., 2010; Anacarso et al., 2011; Lambrecht et al., 2013). As FLP have been isolated from diverse food-related habitats such as broiler houses (Snelling et al., 2005; Baré et al., 2009; Baré et al., 2011), meat cutting plants (Vaerewijck et al., 2008), domestic refrigerators (Vaerewijck et al., 2010) and vegetables (Gourabathini et al., 2008; Vaerewijck et al., 2011), this suggests that FLP may be implicated in the epidemiology of foodborne pathogens.

Many FLP have two life cycle stages: the trophozoite and the dormant cyst. The former is the actively feeding stage, preying on bacteria, algae, viruses, yeast and organic particles by phago- and pinocytosis (Khan, 2006). Encystment (*i.e.* conversion from trophozoite to cyst) is triggered by adverse environmental conditions such as food shortage, hyper- or hypo-osmolarity, temperature and pH extremes (Khan, 2006). Cysts usually possess a thick, often double or multilayered protective wall, consisting of lipids, (glyco)proteins and carbohydrates like chitin and cellulose. This protects the protozoon against unfavorable

environmental conditions such as freezing (Adekambi et al., 2006), γ- and UV-radiation (Aksozek et al., 2002) and chemicals used for disinfection in health care settings (Coulon et al., 2010) and drinking water production (Thomas et al., 2010; Dupuy et al., 2014). Some cysts can withstand desiccation for more than 20 years (Sriram et al., 2008). Under favorable conditions excystment (*i.e*. reconversion into trophozoites) takes place.

To date, most studies on interactions between FLP and bacteria (including pathogenic bacteria) have focused on the trophozoite stage. In contrast, little is known about bacterial association with the cyst forms, which, given their high tolerance for adverse environmental conditions and hence also high dispersal capacity (Khan, 2006), are especially relevant from an ecological and epidemiological point of view. It is expected that FLP cysts are even more effective as a shelter and vector for internalized bacteria than the trophozoites. It has indeed been shown that some internalized digestion resistant bacteria, including human pathogens, can survive the encystment process and may use the cysts as a shelter against harsh environmental conditions (Kilvington and Price, 1990; Adekambi et al., 2006). When environmental conditions become favorable, excystment occurs and internalized bacteria are released (or trigger their release), allowing these bacteria to colonize new habitats (El-Etr et al., 2009).

As FLP can act as a vector and shelter, there is a growing concern that cysts may play a role in the contamination and persistence of pathogenic bacteria in food-related environments. Cysts may thus enable internalized foodborne pathogens to survive physical and chemical cleaning and disinfection methods (Coulon et al., 2010; Dupuy et al., 2014). To date, long term intracystic survival of foodborne pathogenic bacteria isolated from food was never assessed. In order to evaluate the role of FLP in the persistence of foodborne bacterial pathogens, more information is needed on intracellular bacterial survival during the en- and excystment processes and on survival duration inside the FLP cysts. To this end, two strains from each of the five most frequently reported foodborne pathogenic bacteria with a considerable impact on public health and the food industry (EFSA, 2013) were selected, viz. *Campylobacter jejuni*, *Salmonella enterica*, *Escherichia coli*, *Yersinia enterocolitica* and *Listeria monocytogenes*. The amoeba *Acanthamoeba castellanii* was used as a FLP organism, as it is frequently detected in food-related environments (Baré et al., 2009; Vaerewijck et al., 2010).

In the present study, we (i) investigated the uptake/invasion efficiency of these foodborne bacteria by *A. castellanii* trophozoites, (ii) determined the long-term survival capacities of these bacteria inside amoebal cysts using an uniform experimental design; (iii) evaluated the impact of bacteria on protozoan encystment; and (iv) assessed the exact location of these bacteria inside the cysts. To date, survival inside *Acanthamoeba* spp. cysts has been reported for *Mycobacterium spp.* (Adekambi et al., 2006; Ben Salah and Drancourt, 2010), *Francisella tularensis* (El-Etr et al., 2009), *Legionella spp.* (Kilvington and Price, 1990), *Simkania negevensis* (Kahane et al., 2001), *Escherichia coli* (Walochnik et al., 1998; Matin and Jung, 2011), *Vibrio spp.* (Abd et al., 2010), *Enterobacter aerogenes, Aeromonas hydrophila* (Yousuf et al., 2013) and some obligate endosymbionts among which *Protochlamydia amoebophila* (Horn et al., 1999; Collingro et al., 2005). The current study specifically addresses the association mode and the survival duration of the most common foodborne pathogens inside FLP cysts.

3. Material and Methods

3.1 Amoebal strain and culture conditions

Acanthamoeba castellanii (American Type Culture Collection, ATCC30234) was grown axenically in Proteose peptone Yeast extract Glucose medium (PYG, ATCC-recipe, http//www.lgcstandards-atcc.org) at 25°C in 75-cm² tissue culture flasks. Light microscopic observations and plating of culture samples on Plate Count Agar (PCA, Biorad, Hercules, California, USA), which were incubated at 30°C for 48 h, were performed to verify the axenicity of the cultures. After 3.5 days, the amoebae formed a confluent monolayer and were harvested by tapping flasks and subsequent centrifugation of the cell suspensions (300 x g, 5 min). Amoebae were washed with Page's Amoeba Saline (PAS, ATCC-recipe) and suspended in High Saline (HS) buffer (0.1M KCl, 8mM $MgSO_4.7H_2O$, 0.02M Tris, 0.4mM $CaCl_2$, 1mM $NaHCO_3$, pH 9), the medium was also used in further experiments to induce encystment (Ben Salah and Drancourt, 2010). The number of viable trophozoites at the start of each experiment was determined by trypan blue exclusion assays (Lambrecht et al., 2013) using a Füchs-Rosenthal counting chamber (Blaubrand, Wertheim, Germany) and adjusted to $5x10^5$ viable trophozoites/ml HS.

3.2 Bacterial strains and culture conditions

Throughout this study, ten strains belonging to five foodborne pathogenic bacterial species were used: *C. jejuni* isolated from chicken sausage, displaying low invasive properties towards Caco2-cells, and a highly invasive *C. jejuni* isolated from marinated chicken wings (Habib et al., 2009; Baré et al., 2010), *S. enterica* serotype Typhimurium and Enteritidis, both isolated from pig carcasses, enterohaemorrhagic (EHEC) *E. coli* biotype O:157 and O:26 isolated from cattle carcasses, and *Yersinia enterocolitica* bioserotype 4/O:3 isolated from pig carcass and bioserotype 2/O:9 from minced pork meat (Lambrecht et al., 2013), both carrying the virulence plasmid (pYV), and *L. monocytogenes* serotype 4b isolated from dry sausage and 1/2a isolated from salami. All strains were preserved in glycerol at -20°C before use, except for *C. jejuni* which were stored at -80°C in defibrinated horse blood (E&O Laboratories Ltd, Bonnybridge, Scotland). *Campylobacter jejuni* strains were cultivated in

Müller Hinton Broth (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) lysed defibrinated horse blood under micro-aerobic conditions (6% CO_2, 6% H_2, 4% O_2, 84% N_2), while other bacterial strains were cultivated in Tryptic Soya Broth (TSB, Biorad, Hercules, California, USA). For all experiments, all strains were cultivated at 37°C until the stationary growth phase was reached. On the basis of growth curve parameters (data not shown), bacteria were suspended and diluted in HS-buffer to *c.* $5x10^7$ Colony Forming Units (cfu)/ml. To determine the exact number of viable, cultivable bacteria used in the experiments, serial dilutions were plated on PCA, and incubated for 48 h at 30°C, except for *Campylobacter*. For the latter, suspensions were plated on Müller Hinton Agar (MHA, Oxoid, Basingstoke, UK) followed by 48 h incubation at 42°C under micro-aerobic conditions.

3.3 Coculture experiments

All coculture experiments with *A. castellanii* and the ten foodborne bacterial strains were performed in HS-buffer at 25°C. All media and products used during the assays were acclimatized to the desired temperature before the start of the experiments. First invasion/uptake assays were performed to evaluate the bacterial invasion/uptake efficiency by amoebal trophozoites. Then, encystment monitoring assays were performed to assess whether the presence of bacteria influenced the kinetics of the encystment process and *vice versa*. Finally long-term intracystic survival assays and transmission electron microscopy analysis were carried out to assess presence and viability (after excystment) of bacteria inside cysts at different time points.

3.4 Invasion/uptake assays

Invasion/uptake assays were carried out as described previously (Cirillo et al., 1994; El-Etr et al., 2009) with small modifications. *Acanthamoeba castellanii* trophozoites were seeded into $25cm^2$ culture flasks (10 ml/flask at a concentration of *c.*$5x10^5$ cells/ml HS) and incubated at 25°C for 1 h to allow amoebal settlement and adhesion prior to infection. The medium was gently removed and 10 ml bacterial suspension at a concentration of *c*. $5x10^7$ cfu/ml HS was added to each flask to obtain a coculture with a multiplicity of infection (MOI) of *c.* 100

bacteria per amoeba. Bacterial and amoebal monocultures were set up as controls. After 30 min (co)cultivation at 25°C, cells were washed with HS buffer and treated with gentamycin sulphate solution (Sigma-Aldrich, St. Louis, USA) at a final concentration of 100 µg/mL HS buffer for 2 h at 25°C to kill extracellular bacteria. As during the invasion/uptake assays, amoebae were incubated in HS-buffer only for a short time, no conversion to cysts took place. The amoebae were then washed with HS buffer to remove the gentamycin and lysed with 0.5 % sodiumdeoxycholate for 5 min to recover intra-amoebal bacteria. This treatment was effective (100%) in lysing amoebal trophozoites without affecting bacterial viability (data not shown). Cell suspensions of coculture and amoebal monoculture control set-ups were plated on MHA (*Campylobacter)* or PCA (*Salmonella, Escherichia, Listeria, Yersinia*) as described above to determine viable intra-amoebal cfu-counts.

3.5 Encystment monitoring assays

Acanthamoeba trophozoites were infected with bacteria (MOI 100:1, HS buffer) as described for the invasion/uptake assays and incubated at 25°C during six days. Amoebal and bacterial monocultures in HS-buffer were setup as controls. The encystment of *Acanthamoeba* was verified with a light microscope. At d0 and on d1, 2, 3, 4 and 6, viable trophozoites and cysts were counted by trypan blue exclusion assays in a Fuchs-Rosenthal counting chamber. The number of mature cysts was determined by lysis of trophozoites and immature cysts by 3% HCl (Steinert et al., 1998; Ben Salah and Drancourt, 2010). The number of extra-amoebal bacteria during encystment and of bacteria in the monoculture controls was determined by plating serial dilutions on MHA or PCA, as described above.

3.6 Long-term intracystic survival assays

To assess if bacteria were able to survive inside amoebal cysts for longer periods of time (days to weeks), long-term intracystic survival assays were carried out (Fig. 1). Cocultures (combinations of amoebae with a specific bacterial strain) and amoeba and bacterial monoculture controls were setup as described above. Separate culture flasks were used for each excystment time point (see further). After 6 days incubation in HS buffer at 25°C, cells

were treated overnight at 25°C with 3% HCl (pH 0.2) to kill extracellular bacteria, trophozoites and immature cysts. The efficiency of HCl to kill trophozoites was verified by light microscopy. Cells were then washed with PAS-buffer and treated with gentamycin sulphate (100 µg/ml in HS buffer) for 2 h at 25°C to kill any remaining viable extracellular bacteria. Subsequently, cysts were washed, resuspended in fresh HS buffer and incubated at 25°C until the time point for induction of excystment. For the latter, at day 0 (*i.e*. immediately after the gentamycin treatment) and after 1, 3, 7, 14 and 21 days, the cells were washed with PAS and were incubated at 25°C in nutrient rich PYG medium as excystment medium (El-Etr et al., 2009). During excystment, internalized bacteria are released into the medium, where they can further replicate. The process of excystment in both cocultures and amoebal controls as well as the presence of released bacteria in the coculture flasks and of viable bacteria in the bacterial control flasks was checked by light microscopy. Exact quantification of recovered bacteria was assessed at 0h and after 1, 2, 3 and 7 days by plating on MHA or PCA (0.5 ml and serial dilution), as described previously. Enrichment cultures were set up to detect stressed bacteria and/or low bacterial concentrations (enrichment in TSB, ½ Fraser broth for *L. monocytogenes* and MH-broth + 5% (v/v) horse blood for *C. jejuni*, followed by plating on MHA or PCA). The identity of the recovered bacteria was confirmed by conventional biochemical testing (ISO6579-FDAmd1, ISO10273, ISO10272-1, ISO11290-1/A1, ISO16654, www.iso.org).

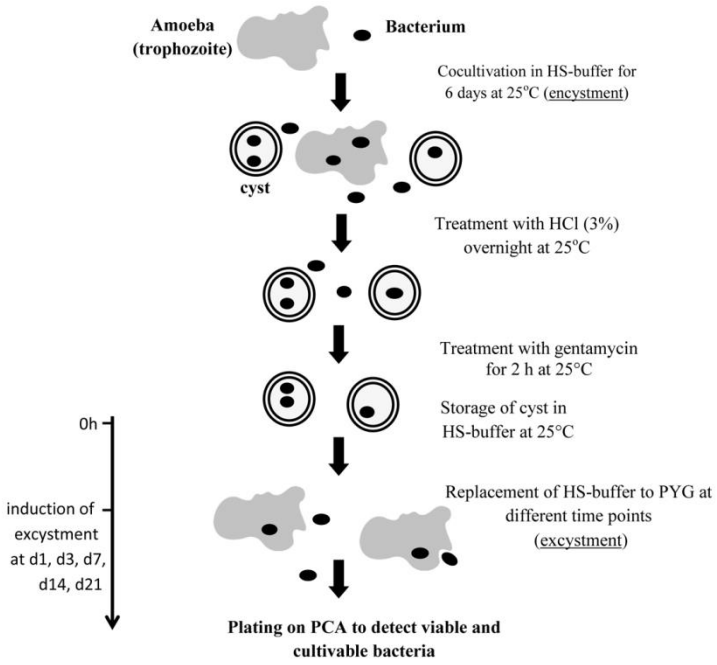

Figure 1. Overview of the experimental setup of the intracystic survival assay

3.7 Transmission electron microscopy

To determine the presence and exact subcellular location of intracystic bacteria, mature cysts, obtained after HCl and gentamycin treatment (see above), were prepared for observation by transmission electron microscopy (TEM). Chemical fixation was performed on collagen coated inserts as described by Lambrecht et al ((Lambrecht et al., 2015), protocol C cf. Chapter III) Briefly, at d0, and on day 3 and 14, cysts were fixed with 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer (pH 7.4) for 2h at room temperature. Subsequently, cells were rinsed with sodium cacodylate buffer, postfixed with osmium tetroxide, dehydrated with ethanol and embedded in Spurr. Sections were stained with uranyl acetate and lead citrate and studied with a Jeol JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) operating at 60 kV. Pictures were digitized using a Ditabis system (Pforzheim, Germany). Per image section, 30 cysts were studied to obtain an indication of the infection ratio (percentage of cysts with intracellular bacteria) and infection intensity (mean number of intracellular bacteria inside bacteria-harboring cysts). Identification of bacteria was based on size and the presence of a double surrounding membrane.

3.8 Data analysis

All experiments were repeated at least three times. Qualitative and quantitative data were recorded in an Excel spreadsheet and statistical analysis was performed on the quantitative data using the software SPSS version 21 (IBM corp., Armonk, New York).

For the invasion/uptake assays, a Wilcoxon Rank Sum test was used to detect differences in entry efficiency between and within species. For the latter, strains of the same bacterial species were clustered. For the encystment monitoring assays, Wilcoxon Rank Sum tests were performed to (i) compare the percentage of cysts between cocultures and amoebal monocultures at each time point and (ii) to compare the absolute numbers of viable, extracellular bacteria between cocultures and bacterial monocultures during encystment.

4. Results

4.1 Foodborne pathogenic strains enter *Acanthamoeba castellanii* with different efficiencies.

Invasion/uptake assays were performed to evaluate the bacterial invasion/uptake efficiencies by amoebal trophozoites before encystment. Results demonstrate that foodborne bacterial pathogens enter the *Acanthamoeba* trophozoite with varying efficiencies (Fig. 2). No viable intra-amoebal listeriae and campylobacters were recovered after 30 min co-cultivation in HS-buffer at 25°C. The invasion/uptake efficiency of the other tested bacteria ranged from 0.0013% to 0.0306%. Significant differences in entry efficiency were observed between but not within species ($p>0.05$). *Escherichia coli* ($p=0.002$) and *S. enterica* ($p=0.002$) exhibited significantly higher invasion/uptake by the amoebae compared with *Y. enterocolitica*. Indeed, after 30 min cocultivation, the invasion/uptake index for both *E. coli* and *S. enterica* was *c.* 1 intracellular bacteria/amoeba, whereas this value was only 0.05 intracellular bacteria/amoeba for the yersiniae.

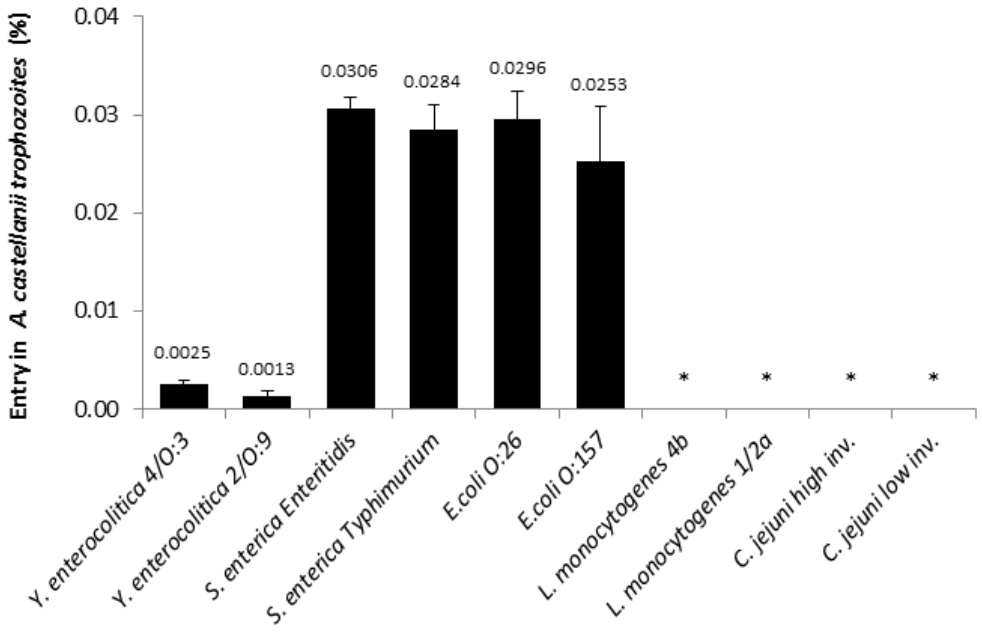

Figure 2. Entry efficiency of different strains belonging to five foodborne bacterial pathogen species in *A. castellanii* trophozoites

Percentage of internalized viable, cultivable bacteria related to the initial inoculum after 30 min cocultivation with *A. castellanii* in HS-buffer at 25°C. Bars represent the mean ± standard error of four replicate experiments. * no viable bacteria could be recovered (detection limit: 1 CFU/ml).

4.2 Presence of bacteria has no impact on amoebal encystment, except with *Y. enterocolitica*

Acanthamoeba is known to encyst in response to adverse environmental conditions including food shortage. To assess if the presence of bacteria, being a potential food source for amoebae, affects the encystment process, encystment monitoring assays were performed. In general, encystment started within 24 h of incubation in HS-buffer. After 2 days, *c.* 54-93 % of the trophozoites, in both cocultures and amoebal monoculture controls, had converted to immature cysts. After 6 days all cysts were mature. With the exception of *Y. enterocolitica* strain 2/O:9 (Fig. 3), no significant differences between cyst percentages in cocultures and monoculture controls could be observed ($p>0.05$). In the presence of *Y. enterocolitica* 2/O:9 (Fig. 3B), cyst percentages were significantly lower compared to amoebal monocultures, for all time points ($p<0.05$). On day 6, only 71 ± 5% (mean ± SEM) of the amoebae were encysted when cocultivated with *Y. enterocolitica* 2/O:9 in comparison to 95 ± 2.5 % in the control condition ($p=0.028$).

In general, the presence of encysting amoebae had no significant influence on the viability of the bacteria ($p>0.05$, data not shown). After 6 days of cultivation the number of viable extra-amoebae bacteria in both cocultures and bacterial monocultures was still *c.* 7 log cfu/ml, except for campylobacters. For the latter, no viable extra-amoebal bacteria could be recovered after 3 days, either in cocultures or bacterial monocultures.

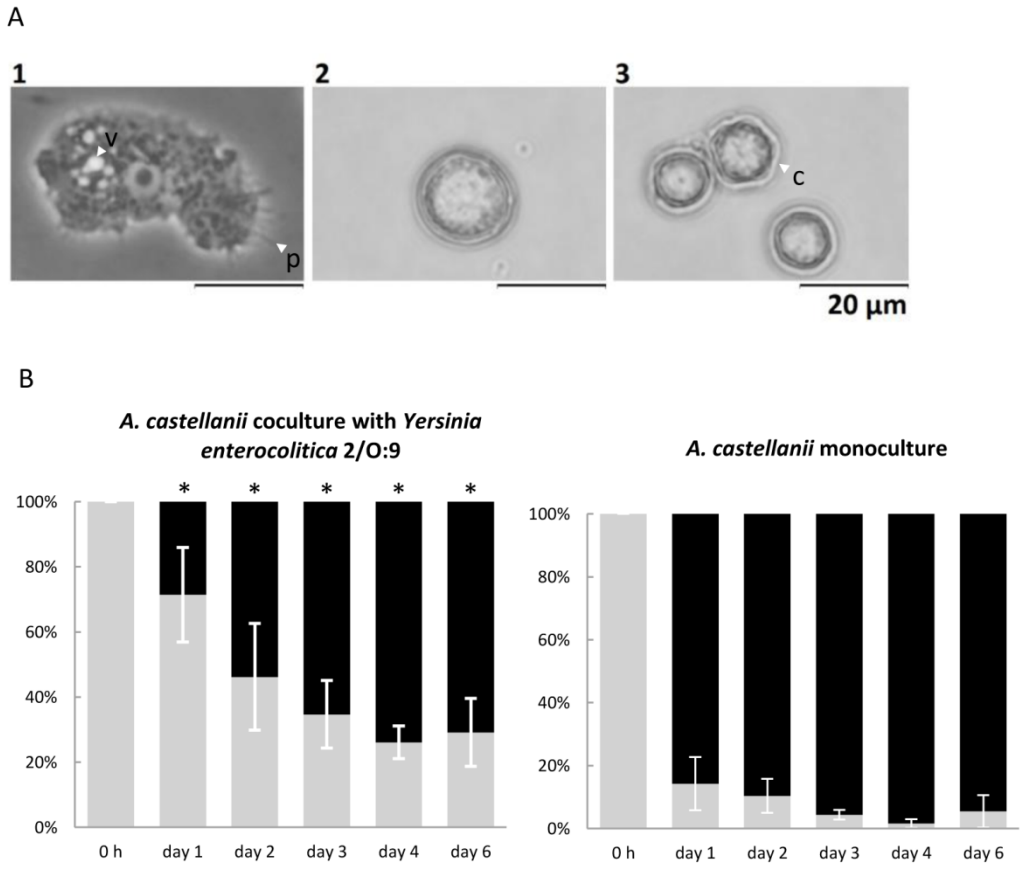

Figure 3. (A) Encystment process of *A. castellanii* (B) *Yersinia enterocolitica* strain 2/O:9 inhibits encystment of *A. castellanii*

(A) *A. castellanii* trophozoite (1), immature cyst (2) and mature cyst (3). Note the presence of pseudopodia (p) and vacuoles (v) in figure A1 and the thick double-layered cell wall (c) in figure A3.

(B) Percentage of *A. castellanii* trophozoites (grey) and (im)mature cysts (black) in the total amoebal count, when cultivated in presence (coculture) or absence (monoculture) of *Y. enterocolitica* at 25°C in HS buffer. Bars represent the mean ± standard error of four replicate experiments. Significant differences ($p<0.05$) between the means of coculture and the corresponding monoculture condition are indicated by *.

4.3 Intracystic survival of foodborne pathogens

The recovery of viable internalized foodborne pathogens from *Acanthamoeba* cysts after periods of days to weeks was species, strain and time dependent (Fig. 4). In general, *S. enterica, L. monocytogenes, Y. enterocolitica,* and *E. coli* could be recovered after induction of excystment, indicating that they were able to survive inside *A. castellanii* cysts. In contrast, cultivable *C. jejuni* could never be retrieved after induction of excystment.

Viable *S. enterica* and *L. monocytogenes* could be recovered from cysts after 21 and 14 days (*i.e.* 27 and 20 days after the setup of the encystment treatment), respectively. In the bacteria monoculture controls, no bacteria were detected, confirming that the HCl- and gentamycin treatment, together with the washing steps, were effective in removing bacteria. Moreover, in none of the cocultures bacteria could be recovered within 24 h after the replacement of the HS buffer with PYG medium, indicating that the bacteria were inside the cysts and not attached to the outer surface of the cyst wall. After 24 to 48 h in PYG medium, excystment occurred and trophozoites and (extracellular) bacteria were visible. After excystment, the extracellular *S. enterica* and *L. monocytogenes* grew rapidly and after 3 days, concentrations up to 9 log cfu/ml were reached. When the bacteria became too dense, the amoebae started to round up, lysed or formed cysts again (data not shown).

Yersinia enterocolitica strains could be recovered from amoebal cysts at excystment time points d0, d1 and d3 by plating on PCA. At later excystment time points, no yersiniae could be detected.

Escherichia coli survived for only a limited time inside cysts. Strain O:26 could be recovered until d3, whereas strain O:157 could only be recovered at d0 (*i.e.* six days after the initial experimental set-up). Three days after induction of excystment, viable extracellular *E. coli* O:26 and O:157 were observed, and from day 5 onwards, extracellular *E. coli* concentrations of 8 log cfu/ml were detected. The trophozoites began to lyse during the first day following excystment (data not shown). However, at later excystment time points (d7-d14-d21), lysis of trophozoites decreased, amoebal trophozoites remained intact and no viable *E. coli* could be recovered.

No viable, cultivable *C. jejuni* were recovered from *A. castellanii* cysts at any time point after induction of excystment, either by direct plating or enrichment, although TEM-images demonstrate the presence of intact intracystic bacteria at d0 and d3 (Fig. 5E).

The viability of extracellular *C. jejuni* already decreased during the encystment stage, as after two days of incubation in HS buffer extracellular *C. jejuni* were coccoid and non-motile, both in cocultures and monocultures.

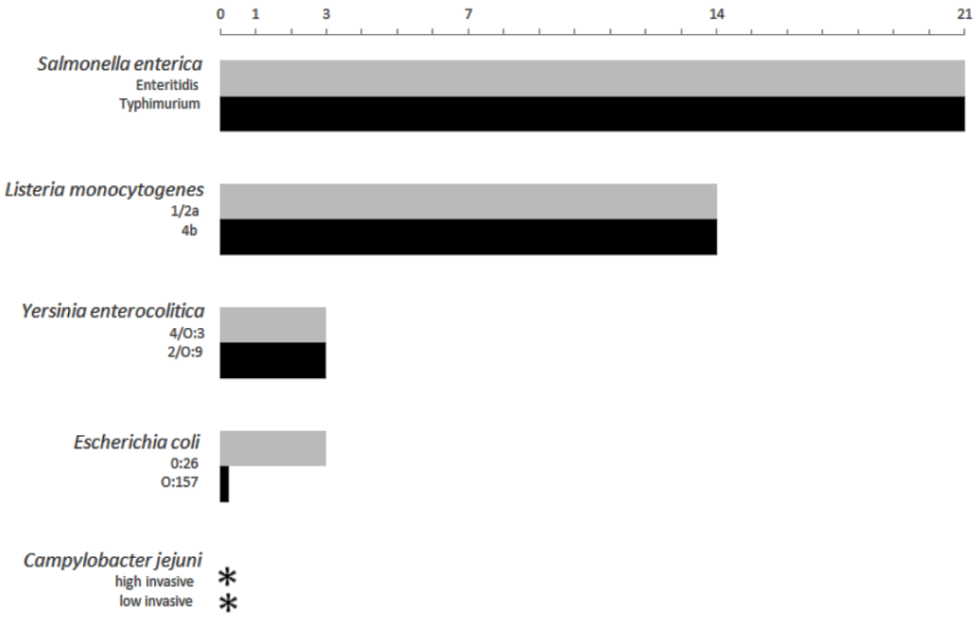

Figure 4. Survival (*S. enterica, L. monocytogenes, Y. enterocolitica, E. coli*) or presence (*C. jejuni*) of foodborne pathogen strains inside *A. castellanii* cysts

Ten strains belonging to five foodborne pathogenic bacterial species were cocultivated with *A. castellanii* trophozoites (MOI 1:100, HS-buffer, 25°C). After 6 days, cysts were treated with HCl and gentamycin and stored in HS-buffer at 25°C (t=0). To detect the intracellular survival of bacteria, excystment was induced at different time points (d0, d1, d3, d7, d14, d21) by replacing HS-buffer with nutrient rich medium. Viability of bacteria was confirmed by colony counting. Bars represent the presence of viable, cultivable bacteria after excystment (n≥3). For each species two strains were tested, indicated by black and grey boxes. * indicates the presence of bacteria inside cysts, as determined by transmission electron microscopy, which were not cultivable after excystment.

4.4 Foodborne pathogens localize inside the cyst cytosol

Transmission electron microscopy was performed to determine the exact intracystic location of the bacteria and to obtain an indication of the infection intensity and ratio. All tested foodborne bacterial pathogens were located in the amoebal cytosol and were not visibly surrounded by an amoebal vacuolar membrane (Fig. 5). At time point 0 h, the percentage of infected cysts (infection ratio) ranged from 17% to 53 % and the mean number of intact bacteria inside cysts (infection intensity) varied between 1.4 and 2.6 (Table 1).

species	0h		d14	
	Infection ratio (%)	Infection intens. (bacteria/cyst)	Infection ratio (%)	Infection intens. (bacteria/cyst)
Salmonella enterica Enteritidis	53	2.6	47	1.4
Salmonella enterica Typhimurium	50	2.4	47	1.5
Listeria monocytogenes 1/2a	37	1.6	27	1.4
Listeria monocytogenes 4b	40	2.2	47	1.2
Escherichia coli O:26	23	1.4	nd	nd
Escherichia coli O:157	17	1.4	0	0
Y. enterocolitica 4/O:3	40	1.6	nd	nd
Y. enterocolitica 2/O:9	40	2.1	0	0
Campylobacter jejuni high invasive	40	2.4	nd	nd
Campylobacter jejuni low invasive	37	2.0	nd	nd

Table 1: Quantification of foodborne pathogenic bacteria inside *A. castellanii* cysts

Data are the result of counts of intact bacterial cells in 30 cysts in one crossection. As such, they only give an indication of the infection ratio (% of infected cysts) and infection intensity (mean number of bacteria inside infected cysts section). nd: not determined, as intracystic survival data indicated there were probably no viable bacteria inside the cysts, no TEM samples were prepared.

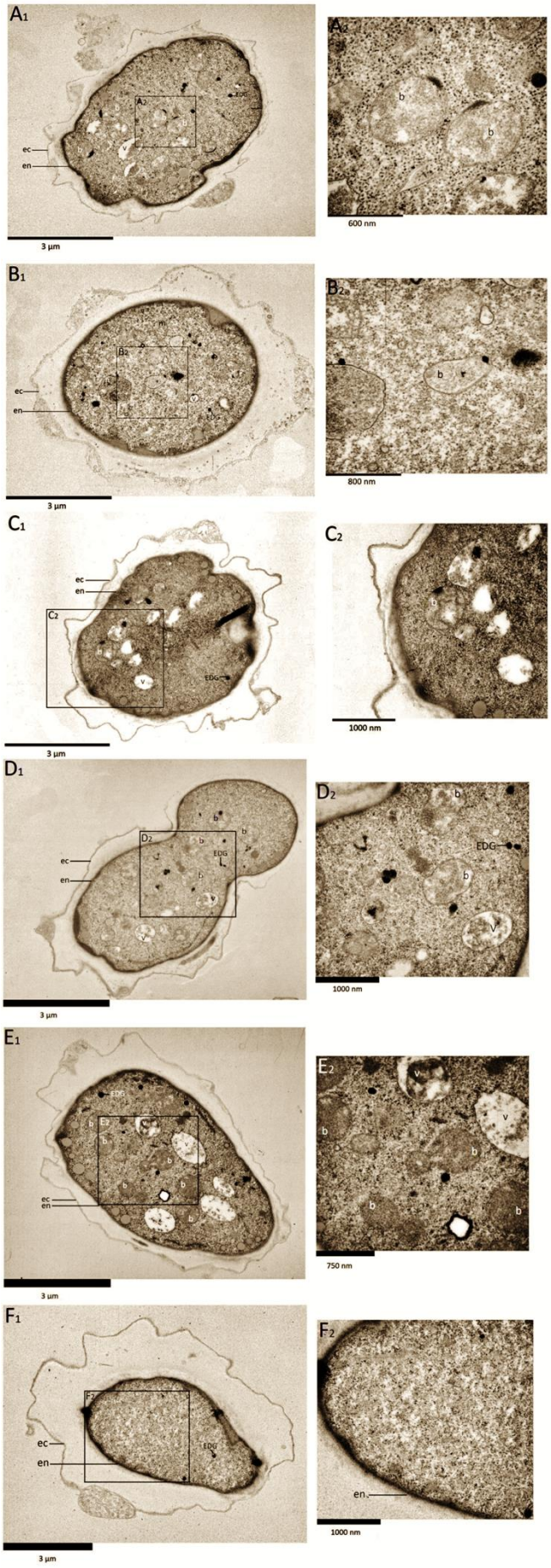

Figure 5. TEM micrographs of *A. castellanii* cyst with internalized bacteria (A-E) and an amoebal monoculture control (F).

A: *S. enterica* subspecies Enteritidis at d0; **B**: *L. monocytogenes* 1/2a at d14*;* **C**: *E. coli O:26* at d0 , **D**: *Y. enterocolitica* 2/O:9 at d0, **E**: *C. jejuni* high invasive towards Caco2-cells at d0.

b: bacteria, ec: ectocyst, en: endocyst, EDG: electron dense granule, n: condensed nucleus, v: vacuole.

5. Discussion

Our study proves that four of the most frequently reported foodborne bacterial pathogens (*S. enterica*, *L. monocytogenes*, *E. coli* and *Y. enterocolitica*) can survive en- and excystment by the ubiquitous amoeba *Acanthamoeba castellanii* and can persist inside the cyst cytosol. While species-specific differences in trophozoite invasion/uptake efficiency were observed, these could not be correlated with the pronounced differences in intracystic survival period, ranging from d0 for *E. coli O:157* up to 21d for both *S. enterica* strains. Moreover, intracystic bacteria were found to be protected against hostile environmental conditions *i.c.* antibiotic treatment (100 µg/ml gentamycin) and low pH (pH 0.2). For all tested species, including *C. jejuni*, the presence of intracystic bacteria was confirmed by transmission electron microscopy. Up to 53% of the cysts contained foodborne pathogenic bacteria. After induction of excystment, *S. enterica*, *L. monocytogenes*, *E. coli* and *Y. enterocolitica* were released or triggered their release into the environment and were able to grow successfully. Bacterial release into the environment can occur through expulsion of bacteria-containing pellets or vesicles from intact amoebal cells or through amoebal lysis (Vaerewijck et al., 2014). Results of the current study could not provide a conclusive answer on the release mechanisms used by the tested bacteria. No culturable *Campylobacter* bacteria, could be recovered from cysts after excystment. Hence identity, viability and culturability of the detected intracystic structures by TEM could not be confirmed. Our results thus show that protozoan cysts can provide internalized foodborne pathogenic bacteria with a shelter against adverse environmental conditions (Kilvington and Price, 1990; Coulon et al., 2010). This sheltering could have significant repercussions on the ecology and epidemiology of foodborne pathogenic bacteria.

To our knowledge, no research has yet been performed to determine the survival capacities of *Salmonella* species in protozoan cysts. We show that *S. enterica* can survive for at least 3 weeks inside *A. castellanii* cysts and preliminary results suggests that they might even survive for up to 3 months inside the cysts (data not shown). Viable *L. monocytogenes* could be recovered from cysts up to 2 weeks. In an earlier study, no viable *L. monocytogenes* could be recovered from *A. castellanii* cysts after 34 days, suggesting that internalized bacteria, if present, may have died within that time frame (Ly and Muller, 1990). Moreover, survival

inside cysts was shown to depend on the *Listeria* strain and *Acanthamoeba* species used (Nale, 2011). Previous experiments demonstrated survival of *L. monocytogenes* in cysts of *Colpoda* spp. (Nadhanan. R, 2012) and *Tetrahymena pyriformis* (Pushkareva and Ermolaeva, 2010). However, some are skeptical about the latter observation as *T. pyriformis* is not known to form cysts (Vaerewijck et al., 2014).

Y. enterocolitica and *E. coli* only survived for up to 3 days inside *A. castellanii* cysts. As two days after inoculation 90 % of the amoeba were already encysted, bacteria recovered at the arbitrary d0-time point (6 days after initial inoculation) were likely to have been encapsulated in the cysts for at least 5 days. No cultivable *Y. enterocolitica* and *E. coli* could be recovered from the cysts at time points later than d3. Transmission electron microscopy confirmed that there were no intact intracystic bacteria present at later time points. This indicates that *Y. enterocolitica* and *E. coli* invade/are taken up by amoebae (cf. confirmed by invasion/uptake assays) and survive for only a limited period of time inside amoebal cysts. Failure to persist for extended periods of time is probably due to intracystic nutrient depletion. The intracystic environment is not an optimal environment for bacteria as during cyst formation, excess food and water is expelled and the protozoon condenses (Khan, 2009)). No data is yet available in the literature regarding intracystic survival of *Y. enterocolitica.* Matin and colleagues (2011) suggested that survival of certain invasive, non-foodborne, *E. coli* strains inside *Acanthamoeba* cysts was possible up to 43 h cocultivation under encystment conditions. As long-term intracystic survival was not tested in this study and a different amoebal encystment method was used, their results cannot reliably be compared with ours.

In contrast to the other tested foodborne pathogens, viable and cultivable *C. jejuni* strains could not be recovered after excystment. A previous study also briefly reported that no *C. jejuni* could be observed in *A. castellanii* cysts (Bui et al., 2012). However, on TEM-images in the present study, intracellular structures, which look like bacteria with an intact cell membrane, were visible in *A. castellanii* cysts. We hypothesize that these structures are bacteria that are in a viable but not cultivable state and cannot be recovered after excystment by plating on MH-agar plates or by enrichment in MH-broth. Another

explanation could be that the intracystic campylobacters do not survive the excystment-process and are rapidly digested by the emerging amoebal trophozoites.

Determination of the exact intracystic location of bacteria was established by transmission electron microscopy. The TEM images of mature cysts at different excystment time points showed that all tested foodborne pathogenic strains were located inside the cyst cytosol. None of the investigated amoebal cross-sections contained bacteria within the double cyst wall. Although these results are novel, bacterial entrapment in the cyst cytosol has already briefly been described before for *Legionella pneumophila* (Kilvington and Price, 1990)*, Vibrio mimicus* and *Simkania negevensis*. The last two species could also be detected within the double cyst walls of *A. polyphaga* cysts (Kahane et al., 2001; Abd et al., 2010).

The species- and strain-specific differences in intracystic survival could not be related to bacterial invasion/uptake efficiency. Remarkably, no viable, cultivable *L. monocytogenes* nor *C. jejuni* could be detected inside trophozoites after 30 min cocultivation in HS-buffer, although they could be detected inside cysts at different time points. Results of the invasion/uptake assays indicate that *L. monocytogenes* and *C. jejuni* prevent their uptake by the trophozoites or were not able to invade them within 30 min. Since later on in the excystment process, viable intracystic bacteria were detected either by cultivation or TEM, it seems that those pathogens indeed need a longer invasion/uptake time. Contradictory information is published about the survival of *Campylobacter* and entry and survival mechanisms of *Listeria* in *Acanthamoeba* (complete overview Vaerewijck et al., 2014)). Differences can be attributed to variations in coculture assays (eg. medium, temperature, multiplicity of infection), and organisms (bacterial strains and protozoan species and strains) (Vaerewijck et al., 2014).

Initially, at least 17 up to 53 % of the cysts were infected with foodborne pathogens, with the minimal estimated infection intensity ranging from 1.4 – 2.6 bacteria per infected amoeba. Moreover, after induction of excystment, all tested bacterial pathogens, with the exception of *Campylobacter*, were able to grow. Currently, the presence of free-living protozoa in food processing plants is not monitored as they are regarded as harmless. Our results, however, suggest that protozoan cysts could be a source of bacterial persistence in the environment and a route for host infection.

Previous studies have shown that bacteria in trophozoites (e.g.King et al., 1988; Whan et al., 2006) and cysts (Kilvington and Price, 1990) were better protected against biocides. This study confirms that in contrast with the free-living bacteria (*cf.* bacteria monoculture controls of the intracystic survival assays), encapsulation of foodborne bacteria inside protozoa cysts protects them against external stresses, *i.c* the gentamycin treatment (100µg/ml) and extreme low pH (pH 0.2, achieved by 3% HCl-treatment). Protection of intracystic bacteria against gentamycin, a commonly used antibiotic in food-animal production (Luangtongkum et al., 2006), has been previously reported and was attributed to the fact that the antibiotic could not pass the double cyst wall (Abd et al., 2010). The sheltering-capacity of protozoan cysts therefore has important food safety and public health implications and could explain why pathogenic bacteria persist in food processing environments, despite thorough disinfection protocols and hygiene monitoring.

Results of the encystment monitoring assays demonstrate that the viability and the encystation process of the amoebae was not markedly affected by intracellular bacteria, except for strain *Y. enterocolitica* 2/O:9. For this strain a significant delay in encystment was observed. This may be due to the fact that a portion of the bacterial population can be used as a food source, making the amoebae less prone to encyst. Another explanation could be that the encystment delay is a result of direct contact between amoebae and bacteria or is mediated by secreted factors. The influence of secreted factors on amoebal encystment has been demonstrated before, as a previous study reported that *Francisella tularensis* caused rapid amoebal encystment by factor(s) secreted by amoebae and/or *F. tularensis* (El-Etr et al., 2009). It is also demonstrated that certain endosymbionts can prevent cyst formation of *Acanthamoeba* and *Hartmannella* (Horn et al., 2000).

In conclusion, we here show that the most common and widespread foodborne pathogenic bacteria can survive in the cytosol of free-living protozoan cysts for extended periods of time. Intracystic survival is associated with increased resistance to adverse environmental conditions (*i.c*. antibiotics and low pH). Pathogenic bacteria may therefore have the potential to use cysts as a vector to colonize new habitats or to infect animal hosts and humans. Moreover, bacteria inside cysts are likely to be undetected by the standardized cultivation protocols for the detection of pathogenic bacteria in food-related environments. Further

research is necessary to identify the factors and conditions which may prevent uptake of foodborne pathogens by free-living protozoa and subsequent protozoan encystation in order to decrease bacterial survival and persistence in food-related environments.

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CHAPTER VI

Impact of amoebal cysts on stress resistance of foodborne bacterial pathogens

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1. Abstract

The formation of robust, resting cysts enables *Acanthamoeba* to resist harsh environmental conditions. This study determined to what extent amoebal cysts are resistant to physical and chemical stresses reflecting procedures commonly used in the food industry. The present study further aims to assess whether intracystic foodborne bacterial pathogens are more stress-resistant compared to bacterial monocultures and if residing inside amoebic cysts induces cross-tolerance towards other stressors once bacteria are released back in the environment.

Several physical and chemical stressors (NaCl, H_2O_2, benzalkoniumchloride, 55 °C, heating until boiling, ethanol, dishwashing detergent and sodium hypochlorite) frequently used in domestic and industrial food related environments, were tested against (i) *A. castellanii* cysts, (ii) bacterial monocultures (iii) intracystic bacteria and (iv) bacteria after intracystic passage (cyst-primed bacteria). Only heating until boiling and hypochlorite treatment were cysticidal. After boiling, no viable trophozoites could be recovered and hypochlorite treatment caused a 1.34 $\log_{10}$ reduction in cyst viability. All treatments were effective in reducing or even eliminating the tested bacterial monocultures. Cyst-primed bacteria however exhibited cross-tolerance towards H_2O_2 and 70% ethanol treatment. Moreover, intracystic passage significantly increased the survival of *Y. enterocolitica, E. coli* and *S. enterica* after NaCl treatment and of *E. coli*, *S. enterica* and *L. monocytogenes* after sodium hypochlorite treatment compared to non-primed bacteria.

These findings underline the potential importance of free-living amoebae in food related environments and their impact in the persistence of foodborne bacterial pathogens.

2. Introduction

Free-living amoebae are ubiquitous in soil, air and water and are part of the in-house microbiota of food related environments. Besides being bacterial predators, free-living amoebae, such as *Acanthamoeba,* can also host grazing-resistant bacteria and are therefore regarded as a reservoir, vector, shelter and a virulence training ground for pathogenic bacteria (Vaerewijck et al., 2014). There is a growing concern that these free-living amoebae contribute to the persistence of bacterial pathogens in food related environments.

Many free-living amoebae exist in two forms: an active trophozoit and a dormant cyst. Nutrient depletion or other environmental stress conditions such as desiccation, or changes in pH, temperature, and oxygen level promote conversion from trophozoites to cysts, a process called encystment. During encystment excess food and water are expelled, and cellular levels of RNA, protein, triacylglycerides and glycogen diminish and the cell volume decreases (Khan, 2009). The cyst structure of *Acanthamoeba,* an amoebal model species and one of the most prevalent free-living protozoa, has been described in detail. The outer wrinkled ectocyst wall is mainly composed of proteins and polysaccharides, while the inner endocyst layer consist of glycans, proteins, fibrils and cellulose (Chavez-Munguia et al., 2005). Although it has been suggested that cellulose is present in both layers of the cyst wall (Chavez-Munguia et al., 2005), other studies propose that cellulose is present only in the endocyst (Weisman, 1976; Linder et al., 2002). The morphology and exact composition of the cyst wall can vary between species and strains, and also depends on the encystation media used (Khan, 2009).

Amoebal cysts are resistant to adverse physical and chemical conditions such as desiccation (Sriram et al., 2008), freezing-thawing cycles, radiation (Aksozek et al., 2002), heat (10 min 80°C, 30 min 70°C, 60 min 60°C, Storey et al., 2004) and various biocides (Turner et al., 2000; Greub and Raoult, 2003; Dupuy et al., 2014). It has been proven that mature *Acanthamoeba* cysts are more resistant than trophozoites to disinfectants and biocides (Khunkitti et al., 1998; Dupuy et al., 2014).

Resistance to harsh environmental conditions plays an important role in the persistence and dispersal of free-living amoebae. There is an increasing concern that free-living amoebae might shelter grazing-resistant internalized bacteria. A pilot scale study on domestic water systems, suggested that *Acanthamoeba* cysts protect internalized *Legionella pneumophila*

from disinfectants as such and were the source of re-colonization after treatment (Thomas et al., 2010).

Resistance can be due to the thick cyst wall, which represents a permeability barrier and/or to the metabolically inactive nature of the cysts, which renders the action of certain biocides ineffective.

Several studies investigated the survival of protozoan trophozoites and cysts after exposure to chemical compounds, such as biocides which are used in water treatment systems and lens disinfectant solutions (Ahearn and Gabriel, 1997; Hughes and Kilvington, 2001; Kilvington and Anger, 2001; Beattie et al., 2003; Dupuy et al., 2014). Although these studies are relevant for prevention and treatment of infections by pathogenic or opportunistic free-living protozoa, these studies did not take into account the fate of the internalized (pathogenic) bacteria. Only a few studies so far have focused on the effect of chemical treatments on intracystic bacteria. *Mycobacterium* has been shown to survive in *Acanthamoeba polyphaga* cysts when exposed to free chlorine (15 ppm, Adekambi et al., 2006). In addition, intracystic foodborne pathogens appeared to be better protected against low pH and gentamycin treatment compared to planktonic bacteria (Lambrecht et al., 2015).

In the current study we investigate the effect of eight stressors, frequently used in domestic and food related environments, on the survival capacities of *Acanthamoeba* cysts and on the foodborne pathogens *Salmonella enterica, Yersinia enterocolitica, Escherichia coli* and *Listeria monocytogenes*, respectively. Further, it was assessed if intracystic located foodborne pathogenic bacteria are better protected against stressful chemical and physical conditions than non-cyst-associated bacteria. In addition, it was evaluated if cyst-primed foodborne pathogenic bacteria, after their release back into the extra-amoebal environment, are more resistant to the various stressors than non-cyst-primed bacteria.

3. Material and methods

3.1 Amoebal strain and culture conditions

Acanthamoeba castellanii (ATCC 30234) was maintained and grown axenically in Proteose peptone Yeast extract Glucose medium (PYG, ATCC-recipe, http//www.lgcstandards-atcc.org)) at 22°C in 75cm^2 culture flasks. To induce cysts formation, the amoebal trophozoites (3.5 days old, forming a confluent monolayer) were resuspended in High Saline buffer (HS-buffer: 0.1M KCl, 8mM $MgSO_4.7H_2O$, 0.02M Tris, 0.4mM $CaCl_2$, 1mM $NaHCO_3$, pH 9) and incubated at 22 °C for 6 days (Lambrecht et al., 2015). Encystment was verified with a light microscope daily. After 6 days, the amoebae were treated for 2 h with 3% HCl to kill immature cysts and any remaining trophozoites. Mature cysts were then resuspended in Page's Amoeba Saline (PAS, ATCC-recipe) and were enumerated with a Füchs-Rosenthal counting chamber (Blaubrand, Wertheim, Germany) and adjusted to 10^5 cysts/ml.

3.2 Bacterial stains and culture conditions

Four foodborne bacterial species, which are able to survive inside amoebal cysts (Lambrecht et al., 2015), were used: *Salmonella enterica, Yersinia enterocolitica, Escherichia coli* and *Listeria monocytogenes* (Table 1). All strains were previously isolated from meat or animal carcasses and stored in glycerol at -20 °C. Bacteria were grown until stationary phase in Tryptone Soy Broth at 37 °C. On the basis of growth curve parameters, bacteria were suspended and diluted in Peptone Water (PW) to obtain the required concentration (c. 2x10^3 Colony Forming Units (cfu)/ ml for the bacterial resistance assay and c. 5x10^7 cfu/ml for the cyst shelter assay). To determine the exact number of viable, cultivable bacteria used at the start of the experiments, serial dilutions were plated on Plate Count Agar (PCA, Biorad, Hercules, California, USA), and incubated 48 h at 30 °C.

Species	Strain ID	Origin
Salmonella enterica serovar Typhimurium	50	Pig carcass
Yersinia enterocolitica 4/O:3, pYV+	5	Minced meat
Escherichia coli O:26, VT2+, EAE+	26	Cattle carcass
Listeria monocytogenes 1/2a	621	Salami

Table 1: Overview of the bacterial strains

3.3. Stressors

Eight stressors, frequently used in domestic and industrial food related environments, were selected on the basis of preliminary studies and previous publications (Table 2).

treatment	final concentration	contact time (min)	company	reference	Chemical/physical stressor
NaCl	5%	120	Sigma	(Gruzdev et al., 2011)	Chemical
H_2O_2	0.3%	15	Laboratoires Gilbert	(Linley et al., 2012)	Chemical
Benzalkoniumchloride	10 mg/L	15	Sigma	(Vaerewijck et al., 2012)	Chemical
55°C water bath	n.a.	15	-	(Coulon et al., 2010; Habib et al., 2010)	Physical
Heating until boiling	n.a.	1 min boiling	-	-	Physical
Ethanol	70 %	5	Chem-Lab, Disolol	(Coulon et al., 2010)	Chemical
Dishwashing detergent*	0.8 ml/L	5	Proctor & Gamble	-	Chemical
Sodium hypochlorite	2.5%	15	17% free chlorine, VWR, dissolved in tap water	(Coulon et al., 2010)	Chemical

Table 2: Overview of the stressors tested in the this study, their concentration and their contact times
Unless stated otherwise, treatments were performed at 22°C.
* Commercial dishwashing detergent containing 15-30% aniongenic surface active molecules, 5-15% non-iongenic surface molecules, methylisothiazolinone, phenoxyethanol and perfume

3.4 Cysts resistance assay

Eight stressors (Table 2) were tested for their cysticidal effect on *A. castellanii* cysts by using the most probable number (MPN) technique for amoebic enumeration. This technique is simple and reproducible and was performed as described by Beattie et al. (2003) with some modifications. In short, 1 ml aliquots of cysts cultures (10^5 cysts/ml) were added to 9 ml of each chemical stressor (concentration see Table 2) or in 9 ml PAS for the physical stress treatments and the controls. All tested solutions were freshly prepared. After exposure to the various stress treatments, the stressors were neutralized by adding 1 ml of stressed cysts

suspension to 9 ml PAS or appropriate neutralizer (1:10 dilution). Benzalkonium chloride was neutralized in 30g/l Tween 80 and 3g/l soy lecithin dissolved in dionized water (Vaerewijck et al., 2012). Sodium hypochlorite was neutralized in 30g/l Tween 80 and 5g/l sodium thiosulphate in dionized water (Vaerewijck et al., 2012). Afterwards, cysts were washed with PAS and resuspended in the same amount of PYG. This 1:10 suspension, was serially diluted in PYG to obtain a $1:10^2$, $1:10^3$ and $1:10^4$ dilution. Five replicates of 1 ml of each dilution were inoculated in 24-well plates. The plates were incubated under aerobic conditions at 22°C in the dark. The plates were examined for the presence of viable trophozoites after 2 days, 7 and 14 days incubation. Plates were considered as positive (score 1) when adherent, moving amoebal trophozoites with distinct vacuoles and nucleus were present. Absence of these trophozoites was considered as negative and scored as 0. The score for each 10 fold-dilutions (i.e., 10^{-2}, 10^{-3}, 10^{-4}) then yields a three digit number, which is then entered into the MPN table to give the most probable number of amoebae per ml (Beattie et al., 2003).

3.5 Bacterial resistance assay

Chemical and physical stressors (Table 2) were tested for their bactericidal effect on monocultures of foodborne bacteria. Therefore, bacterial cultures were aliquoted (500 µl, $2x10^3$ cfu/ml PW) in Eppendorf tubes and immediately mixed with 500 µl of double strength chemical stressor solution to achieve the indicated concentration in a final volume of 1 ml with 10^3 bacteria/ml. This bacterial concentration is representative for the number of intra-amoebal bacteria (Lambrecht et al., 2013, Lambrecht unpublished). For physical stressors (heating until boiling and 55°C) and for the non-treated controls, bacteria were mixed with 500 µl H_2O. The cells were gently vortexed and incubated in the presence of the stressors for the indicated times (Table 1). After exposure, bacterial cells were neutralized by 10-fold serial dilution in PW and viable bacteria were enumerated by plating on PCA. Benzalkoniumchloride and sodium hypochlorite were neutralized with a Tween 80 based neutralizer (see 3.3) before serial dilution in PW and plating on PCA. In addition, 100 µl of the neutralized, undiluted sample was enriched by inoculation in 9 ml PYG (excystment medium) and incubated at 22°C for 3 days before plating on PCA to detect low numbers of viable bacteria. Plates were incubated at 30°C for 48 h to enumerate the bacteria. The

identity of the recovered bacteria was confirmed by conventional microbial testing (ISO6579-FDAmd1, ISO10273, ISO10272-1, ISO11290-1/A1, ISO16654, www.iso.org).

3.6 Cyst shelter assay

To assess if amoebal cysts can protect internalized bacteria against chemical and physical stressors, a cyst shelter assay was set up. Briefly, bacteria ($5x10^7$ cfu/ml) were coincubated with $5x10^5$ *Acanthamoeba* trophozoites/ml (multiplicity of infection 1:100) for 6 days in HS-buffer at 22°C in 25cm^2 culture flask (Lambrecht et al., 2015). Under these conditions, amoebal trophozoites will take up bacteria and start to encyst. Monocultured, axenic amoebae were used as controls. The formation and morphology of cysts was checked microscopically. After 6 days, adherent cells were washed with PAS and subsequently treated with 3% HCl to remove immature cysts and remaining trophozoites and to reduce extracellular bacteria. Next, the mature cysts (c. 10^5 cysts/ml) were treated with gentamycin 100µg/ml for 1 h at 22°C to kill any remaining extracellular bacteria, washed twice with PAS and challenged with various stressors (Table 2). Afterwards, cells were neutralized as described in 3.2, washed in PAS and resuspended in PYG. The culture flasks were incubated at 22 °C and checked daily by light microscopy for the presence of excysted amoebae. Before incubation and on day 1, 2, 3, 5,7 and 14, a fraction of the supernatant was serially diluted and plated on PCA and incubated at 30 °C for 2 days to detect released, extracellular bacteria. The identity of the recovered bacteria was confirmed by conventional microbial testing as described above.

3.7 Cyst-induced resistance assay

To evaluate if bacterial survival inside amoebic cysts induces enhanced bacterial tolerance to other stressors once intracystic bacteria are released back in the environment, cyst-induced resistance assays were performed. To obtain intracystic bacteria, cocultures of amoebal trophozoites and bacteria in HS-buffer were set up as described above for the cyst shelter assay. After HCl and gentamycin treatment, cysts were washed twice with PAS, resuspended in PYG and incubated at 22 °C. After 24 to 48 h incubation, excystment occurred by which trophozoites and released, extracellular, cyst-primed bacteria (c. 10^3 cfu/ml) were visible.

Bacterial non-primed control suspensions were set up by inoculating 10^3 cfu/ml glycerol stock bacteria in PYG. Both primed and non-cyst primed bacteria were incubated for 3 more days to reach the stationary phase for all bacteria (c. 10^8-10^9 cfu/ml). Afterwards, a fraction of each suspension was serially diluted and plated on PCA for bacterial enumeration ($N_{\text{before treatment}}$). The remaining fraction was treated with the various stressors for the indicated times and neutralized as described above. After treatment, the cells were washed and resuspended in PW. The suspensions were serially diluted, plated on PCA and incubated at 30°C for 2 days to enumerate bacteria ($N_{\text{after treatment}}$). The identity of the recovered bacteria was confirmed by conventional microbial testing as described above.

3.8 Statistical analysis

All experiments were repeated at least four times. Quantitative data on bacterial survival and estimated MPN values for cysts were recorded in an Excel spreadsheet and statistical analysis was performed on the Log_{10} transformed quantitative data using the software SPSS version 21 (IBM corp., Armonk, New York).

For the cysts and bacterial resistance assays, a Wilcoxon Rank Sum test was used to detect differences in the median Log_{10} reduction between cells that have been treated with stressors and the untreated controls

For the cyst induced resistance assay, a Wilcoxon Rank Sum test was used to detect differences in median Log_{10} reduction after stress treatment ($N_{\text{before treatment}}$- $N_{\text{after treatment}}$) between bacteria that have been associated with amoebal cysts and the monoculture bacteria controls.

4. Results

4.1 Cysticidal effect of chemical and physical stress treatments

Cysts of amoebal monocultures were treated with various chemical and physical stress factors (Table 1). Only heating until boiling and sodium hypochlorite treatment demonstrated cysticidal[1] activity that was significantly different from the reductions in the non-treated controls. After heating until boiling, no viable trophozoites could be recovered. Sodium hypochlorite treatment caused a 1.34 log_{10} reduction in cyst viability by day 14. For all other treatments, no significant reduction in amoebal viability could be observed by day 14. However, 55°C and 70% ethanol treatment caused a delay in excystment, as after two days a 2.22 log_{10} and 4.47 log_{10} reduction were observed respectively, which was significant higher compared to the non-treated controls. This delay was no longer present on day 7. Cysts challenged with the stressors did not differ morphologically (light microscopy) from the controls.

	day 2		day7		day 14	
	M	IQR	M	IQR	M	IQR
Treatment condition						
5% NaCl, 120 min	2.02	[2.02-3.37]	1.10	[1.01-1.7]	0.46	[0.43-0.60]
0.3% H_2O_2, 15 min	1.47	[1.37-4.72]	1.18	[1.18-1.18]	0.46	[0.41-0.75]
10 mg/l Benzalkoniumchloride, 15 min	1.60	[1.54-1.60]	1.18	[0.82-1.18]	0.46	[0.41-0.60]
55°C, 15 min	2.22	[2.22-2.37]	0.51	[0.51-1.39]	0.46	[0.41-0.60]
Heating until boiling, 1 min	4.72	[4.72-4.72]	4.72	[4.42-4.72]	4.78	[4.66-4.86]
70% ethanol, 5 min	4.47	[4.47-4.47]	2.27	[2.20-2.39]	0.48	[0.41-1.11]
0.8ml/L dishwashing detergent, 5 min	1.37	[1.32-1.47]	1.18	[1.18-1.22]	0.46	[0.41-0.96]
2.5 % sodium hypochlorite, 15 min	4.72	[4.72-4.72]	2.27	[1.80-2.2]	1.34	[1.28-1.50]
Control condition						
Non-treated controls	0.91	[0.87-0.98]	0.52	[0.41-0.60]	0.46	[0.39-0.51]

Table 3: Median Log_{10} reduction (M) in cyst viability after treatment with various stressors
IQR: interquartile range, N=4, range, gray shaded box= significant ($p<0.05$) reduction compared to non-treated controls.

[1] a cysticidal treatment is defined as a treatment that resulted in significant lower excystment after 14 days of incubation in excystment buffer

4.2 Amoebal cysts shelter internalized bacteria against various stressors

In the bacterial resistance assay, bacterial monocultures were treated with various chemical and physical stress factors. In general, for each bacterial strain, all treatments were effective in reducing or even eliminating the monocultured bacteria ($p<0.05$, Figure 1). Treatment with 0.3% H_2O_2, 10 mg/L benzalkoniumchloride, 70% ethanol, 2.5 % hypochlorite and incubation at 55°C and heating until boiling led to a total bacterial elimination (i.e. no viable bacteria could be detected, detection limit: 1cfu/ml) for all tested strains. Dishwashing detergent and 5% NaCl were less bactericidal, and species differences were observed. *Yersinia* (0.12 Log_{10} reduction) and S*almonella* (0.05 Log_{10} reduction) were significantly more resistant towards 5% NaCl than the other tested pathogens (c. 1.5-2 Log_{10} reduction). Dishwashing detergent caused a significant reduction (c. 1-1.5 Log_{10} reduction) in bacterial numbers, but no significant differences between species were observed.

Bacteria inside amoebal cysts were protected against the majority of the stressors (Figure 1). Except from cyst-associated bacteria incubated at 55 °C or heated until boiling, all tested bacterial strains could be recovered from the cysts after the various stress treatments. After induction of excystment, trophozoites and extracellular bacteria started to appear within 24-48 h. Released bacteria were able to grow in presence of the trophozoites.

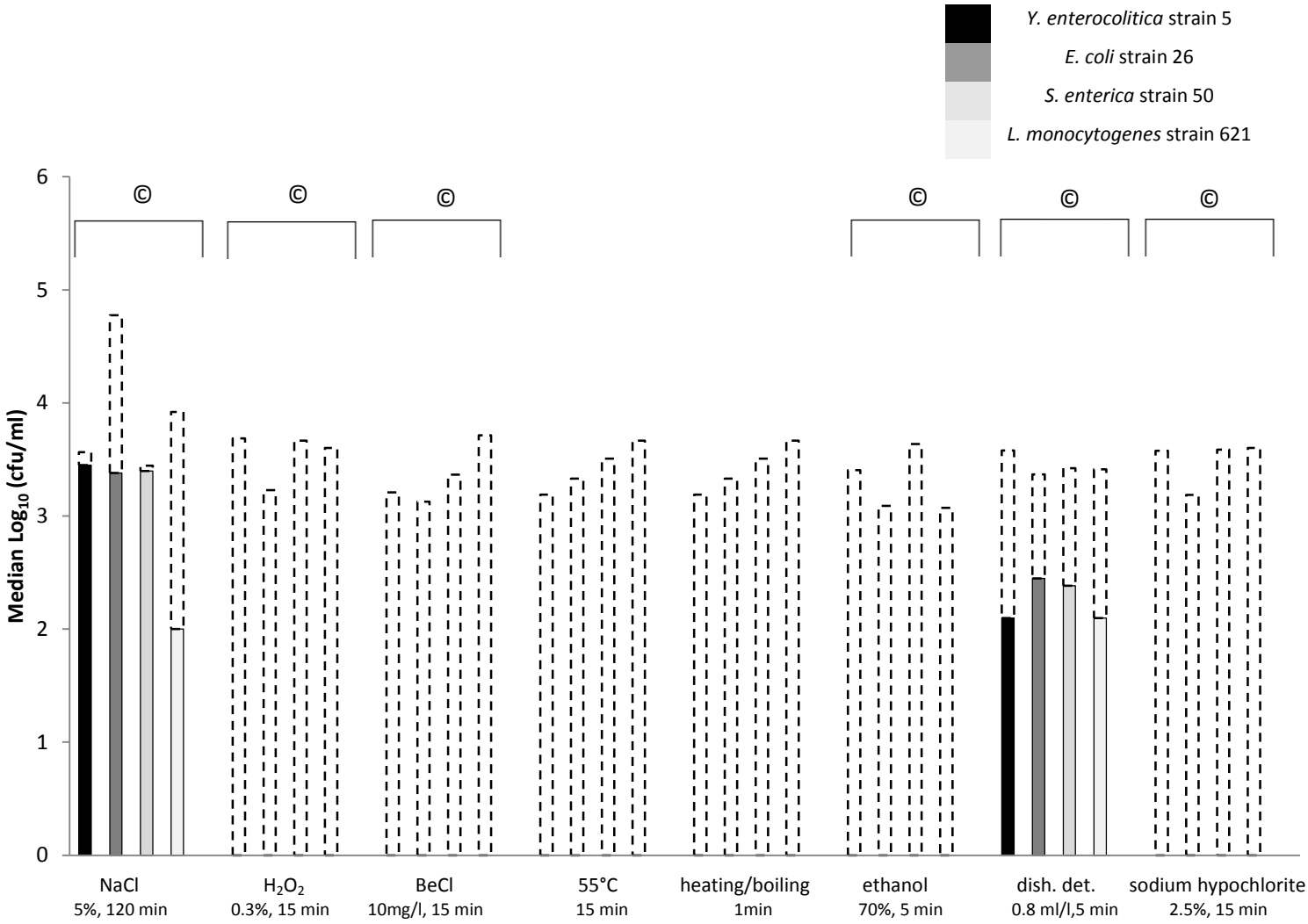

Figure 1 Efficacy of various stress treatments on foodborne pathogens and sheltering capacity of *A. castellanii*

Median numbers of monocultured bacteria without treatment (controls, dashed lines) and of monocultured bacteria after stress treatment (shaded boxes). BeCl= benzalkoniumchloride, dish. det: dishwashing detergent, N= 4

©= viable bacteria could be recovered from cysts after treatment

4.3 Priming inside amoebal cysts provides higher stress tolerance after release

To evaluate if intracystic passage (cyst-priming) induces enhanced tolerance to other stressors once bacteria are release back in the environment, cyst-induced resistance assays were performed. Before treatment, the bacterial concentrations of primed and non-primed bacteria were between c. 10^8-$5x10^8$ cfu/ml. Cyst-primed bacteria acquired a higher tolerance to multiple stressors compared to the non-cyst-primed cells (Figure 2). For all tested bacteria, a lower reduction in bacterial numbers was observed in primed-bacteria when exposed to 0.3% H_2O_2 and 70 % ethanol treatment ($p<0.05$). Moreover, cyst passage significantly increased ($p<0.05$) the survival of *Y. enterocolitica, E. coli* and *S. enterica* after NaCl treatment and of *E. coli*, *S. enterica* and *L. monocytogenes* after sodium hypochlorite treatment.

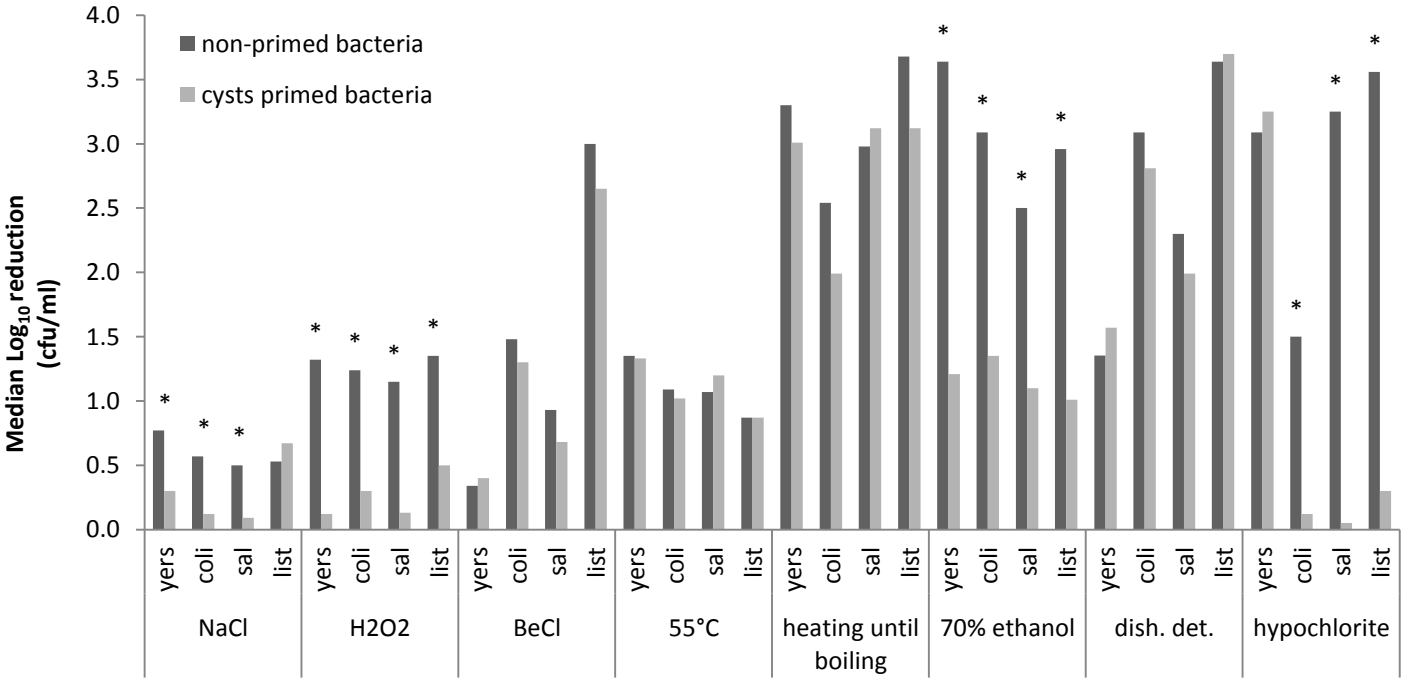

Figure 2 Effect of stressors on the survival of monocultured bacteria (non-primed) and primed bacteria after intracystic passage.
Yers= *Yersinia enterocolitica* strain 5; coli = *Escherichia coli* strain 26, sal= *Salmonella enterica* strain 50, list= *Listeria monocytogenes* strain 621, BeCl= benzalkoniumchloride, dish. det: dishwashing * P< 0.05 between primed and non-primed bacteria detergent, N=4

5. Discussion

Free-living amoebae are widespread in the environment including domestic and food-processing environments (Vaerewijck et al 2014). Their role as protective and dissemination host for other (pathogenic) microorganisms makes them a potential risk to human health, and food safety in particular. Like many free-living amoebae, *Acanthamoeba* is able to form cysts, which have been shown to be resistant to various biocides used in water purification and contact lens disinfectants (Beattie et al., 2003; Dupuy et al., 2014). Moreover, *Acanthamoeba* appears to be a universal free-living protozoan host used by the majority of pathogenic bacteria identified to date (Loret and Greub, 2010), but this finding is probably biased because of its widespread use as a model organism.

The goals of the present study were to asses if intracystic foodborne pathogenic and cyst-primed bacteria are better protected against stressful treatments than non-cyst-associated bacteria. Therefore, foodborne pathogens (as monocultures, located within amoebal cysts or passaged through cysts) were subjected to various stressors. The treatment doses were chosen to be representative for actual cleaning and disinfection practices in domestic and industrial food related environments.

The results of the bacterial resistance assay clearly illustrate that monocultured bacteria are vulnerable towards the tested stressors (NaCl, H_2O_2, benzalkoniumchloride, 55 °C, heating until boiling, ethanol, dishwashing detergent and sodium hypochlorite), confirming the usefulness of those treatments in food related environments.

The results of the cyst resistance assay demonstrated that heating until boiling and treatment with 2.5 % sodium hypochlorite for 15 min was effective in killing amoebal cysts. Heat causes denaturation of the protein constituents as well as liquefaction of the microbial membranes (Mackey et al., 1991). The effectiveness of boiling has already been documented by Coulon et al 2010, who observed a >4 Log_{10} cyst-reduction in all tested *Acanthamoeba* strains. Moreover, in accordance with our results, these authors demonstrated that exposure at 55 °C for 10 min is inefficient in reducing cyst viability. Although heating until boiling seems to be an easy, cheap and effective procedure to eliminate amoebal cysts, it should be noted that there are reports of some thermotolerant strains which could resist exposure to 80 °C for 10 min (Storey et al., 2004).

Sodium hypochlorite, or household bleach, is known to have a broad spectrum of antimicrobial activity and is already widely used in the food industry for e.g. for sanitizing food contact surfaces. Effectiveness of hypochlorite in killing *Acanthamoeba* cysts (Beattie et al., 2003; Critchley and Bentham, 2009; Coulon et al., 2010), has been confirmed in the present study. The microbiocidal activity of chlorine is mainly attributed to undissociated hypochlorous acid, but the exact mechanisms by which it destroys microorganisms is as yet unknown. Inactivation could result from a number of factors including, nucleic acid denaturation, oxidation of respiratory components and impairment of enzyme and protein functions (Stewart and Olson, 1996). Despite its effectiveness in eliminating bacteria and cysts, hypochlorite treatment has some disadvantages, including corrosiveness to metals, inactivation by organic matter, and release of toxic chlorine gas when mixed with ammonia or acid. Hypochlorite can cause swelling of the cysts wall, due to interactions of the biocide with the inner cysts wall (Coulon et al., 2010). However, In the present experiments, no swelling of the cysts was observed by light microscopy.

Remarkably, also for the non-treated control cysts a small reduction in amoebal numbers was observed. This can probably be explained by loosening and subsequent loss of cysts during the various washing steps. The other tested stress treatments did not have a significant cyst reducing effect compared to the controls, confirming that *Acanthamoeba* cysts are robust entities.

Tolerance towards these stresses might be caused by metabolic inactivity of the stressor by the amoebal cyst or a decreased uptake/diffusion of the stressors. The cyst wall can be considered as a mechanical barrier and composition and thickness can be related to the degree of stress tolerance. A study performed with *Acanthamoeba* cysts revealed strain differences among various treatments, with the most resistant strains exhibiting a thicker ectocyst structure (Coulon et al., 2010). Dupuy and colleagues (2014) observed that *Acanthamoeba* cysts were less sensitive to chlorine than *Hartmannella* cysts. The authors stated this might reflect the difference in cellulose content between those species, as *Hartmannella* has only a small quantity of cellulose in the envelope (4.2%) compared to *Acanthamoeba* (30%).

Kilvington and Anger (2001) showed there was an effect of the cyst age on disinfectant efficiency, with mature cysts being more resistant than immature cyst.

Moreover, it should be underlined that resistance to treatments can depend on the applied encystment induction method (Hughes et al., 2003; Coulon et al., 2012). These findings make it difficult to compare cysts resistance values between different studies.

Bacteria inside amoebal cysts were more tolerant to stressful conditions. Whereas monocultured bacteria were killed after H_2O_2, 70 % ethanol and 2.5 % hypochlorite treatments, those that had been inside cyst survived the same treatments. Hypochlorite treatment was demonstrated to be cysticidal, suggesting that non-viable cysts could still protect internalized bacteria against this treatment. Cysts failed to protect internalized bacteria when incubated at 55°C or heated until boiling, indicating that cysts mainly provide a physical barrier against chemicals.

Before excystment of the amoebae (i.e. 24-48h after inoculation in PYG), no extracellular bacteria were observed, indicating that bacteria recovered at time points after excystment were indeed from inside the cysts. The exact amount of bacteria inside the cysts was not determined, as the robust cyst wall might hamper staining of viable intracellular bacteria. The presence of viable bacteria after excystment implies that at least a part of the bacteria were also viable inside the cysts.

The results of the cyst induced resistance assay revealed that bacterial passage through cysts induces tolerance to various stressors. It has been described that exposure to one stressor renders the bacteria resistant to other subsequent stress factors (cross-resistance, (Vorob'eva, 2004; Kultz, 2005). The intracystic environment can be considered as a stressful condition as cysts dehydrate and expel nutrients during encystment (Khan 2009). Bacteria, including foodborne pathogens, have evolved complex interacting systems to tolerate desiccation and osmotic stresses (Gruzdev et al., 2011; Burgess et al., 2016). Gruzdev proved that desiccated *Salmonella* acquired higher tolerance to subsequent ethanol, hypochlorite, hydrogen peroxide and NaCl treatment (Gruzdev et al., 2011). This is in concordance with the results obtained in the current study. Cross-protection against several stressors was also observed for *Y. enterocolitica*, *L. monocytogenes* and *E. coli*, suggesting the presence of a common stress response mechanism for this bacterium that is responsible for the development of cross-tolerance to other stressors. Further research is needed to reveal how long this increased tolerance last and how we can counteract/prevent it.

It was not in the scope of this study to determine the minimal effective dose and treatment time to kill cysts and bacteria. This would imply testing different durations and concentrations of stressors on various bacterial strains and in interaction with multiple amoebal strains. Others have already demonstrated that inhibitory concentrations are highly variable among amoebal strains and culture conditions (Hughes et al., 2003). To fully assess the risk of free-living protozoa in food related environments, standardized stress-tolerance protocols and improved isolation and cultivation techniques for 'in-house-specific' amoebae, ciliates, flagellates and cysts need to be developed.

In the present study, we proved that *Acanthamoeba* can induce cross-tolerance in foodborne pathogenic bacteria *Y. enterocolitica*, *S. enterica*, *E. coli* and *L. monocytogenes* against stressful chemical and physical conditions, which are frequently used in domestic and industrial food related environments. The ability to survive and/or proliferate under stresses is well known to contribute to the persistence of pathogens in foods and food-processing environments, elevating the risk of pathogen transmission through the food chain to humans.

The strong, protective association between FLA and foodborne pathogens demonstrated in this study, underscores that reducing or eliminating foodborne pathogens also requires a better inventory and control of FLA in food related systems.

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CHAPTER VII

General discussion and future perspectives

	FLA can act as				Survival in cysts	Beneficial extracellular association
	Reservoir	Vector	Shelter	Training ground/ cross resistance		
Campylobacter jejuni	V (Axelsson-Olsson 2005, 2013, Snelling 2005,2008, Nguyen 2011, Olofsson 2013)	V (Snelling 2005,2008) X **(Lambrecht 2015)**	V (King 1988, Snelling 2005, Axelsson-Olsson 2010) X **(Lambrecht 2015)**		X **(Lambrecht 2015)**	V (Snelling 2005, Baré 2010, Bui 2012)
Salmonella enterica	V (Gaze 2003,Hadas 2004, Anacarso 2011)	V (Gaze 2003, Hadas 2003, **Lambrecht 2015)**	V (Brandl 2005, **Lambrecht 2015)**	V (Rasmussen 2005,McCuddin 2006, Xiong 2011, **Lambrecht 2016)**	V **(Lambrecht, 2015)**	V (Gaze 2003, Huws 2008, Bui 2012, Douesnard-Malo 2011)
Yersinia enterocolitica	V (Anacarso 2011, **Lambrecht, 2013)**	V **(Lambrecht, 2015)**	V (King 1988, **Lambrecht 2015)**	V **(Lambrecht, 2016)**	V **(Lambrecht, 2015)**	
Verocytotoxigenic *Escherichia coli*	V (Barker 1999, Chekabab 2012)	V **(Lambrecht, 2015**	V **(Lambrecht 2015)**	V **(Lambrecht, 2016)**	V (Matin 2011, **Lambrecht 2015**)	
Listeria monocytogenes	V (Ly and Muller 1990, Anacarso 2011) X (Zhou 2007, Huws 2008, Akya 2009,2010, Fieseler 2014)	V **(Lambrecht, 2015)**	V (Nadhanan & Thomas 2014, **Lambrecht 2015**)	V **(Lambrecht, 2016)**	V **(Lambrecht, 2015)** X (Ly 1990)	V (Huws 2008, Zhou 2007, Fieseler 2014)

Table 1. Overview of major studies that confirmed interactions between FLA and several foodborne pathogens.

All studies were performed *in vitro* unless indicated otherwise. *in situ* study, *in vivo* study

V: tested and advantageous role of FLA confirmed, X: tested but not confirmed

reservoir: the amoeba act as a host, wherein bacteria are able to grow (Brown and Barker, 1999)

vector: the amoeba act as a vehicle which actively (trophozoites) or passively (cyst) spreads the internalized bacteria (Anacarso et al., 2011)

shelter: the amoeba can protect the internalized bacteria against stressfull environmental conditions

training ground/cross resistance: the amoeba can increase bacterial virulence or increase persistence against stressfull conditions (Molmeret et al., 2005)

beneficial extracellular association: bacteria benefit from an extracellular amoeba association (eg. by using secreted amoebal metabolites)

1. Research positioning & scientific contributions

Despite strict control measures to reduce or eliminate bacterial foodborne pathogens from domestic and food-related environments, recurrent contaminations and/or infections with these pathogens still occur. This suggests an uncomplete knowledge of pathogen ecology and epidemiology, which makes design of effective control measures difficult. Free-living amoebae, such as *Acanthamoeba*, *Hartmannella* and *Vannella* form a natural component of the in-house flora of food-related environments and can contribute to the survival, protection and dissemination of bacteria (Baré et al., 2009; Baré et al., 2011; Vaerewijck et al., 2014; Chavatte, 2016). Therefore, FLA are identified as a possible avenue by which pathogens can resist cleaning and disinfection procedures. Although it has been shown that foodborne pathogens and free-living protozoa co-occur in the same niches, the interaction between FLA and foodborne bacterial pathogens (*i.e. C. jejuni, S. enterica, Y. enterocolitica, E. coli* and *L. monocytogenes)* had not been well documented to date (for overview, including the results of this PhD study, see Table 1). This doctoral research furthers the knowledge and assessed the potential risks of the interaction of these foodborne pathogens with FLA using *in vitro* cocultivation assays (Figure 1).

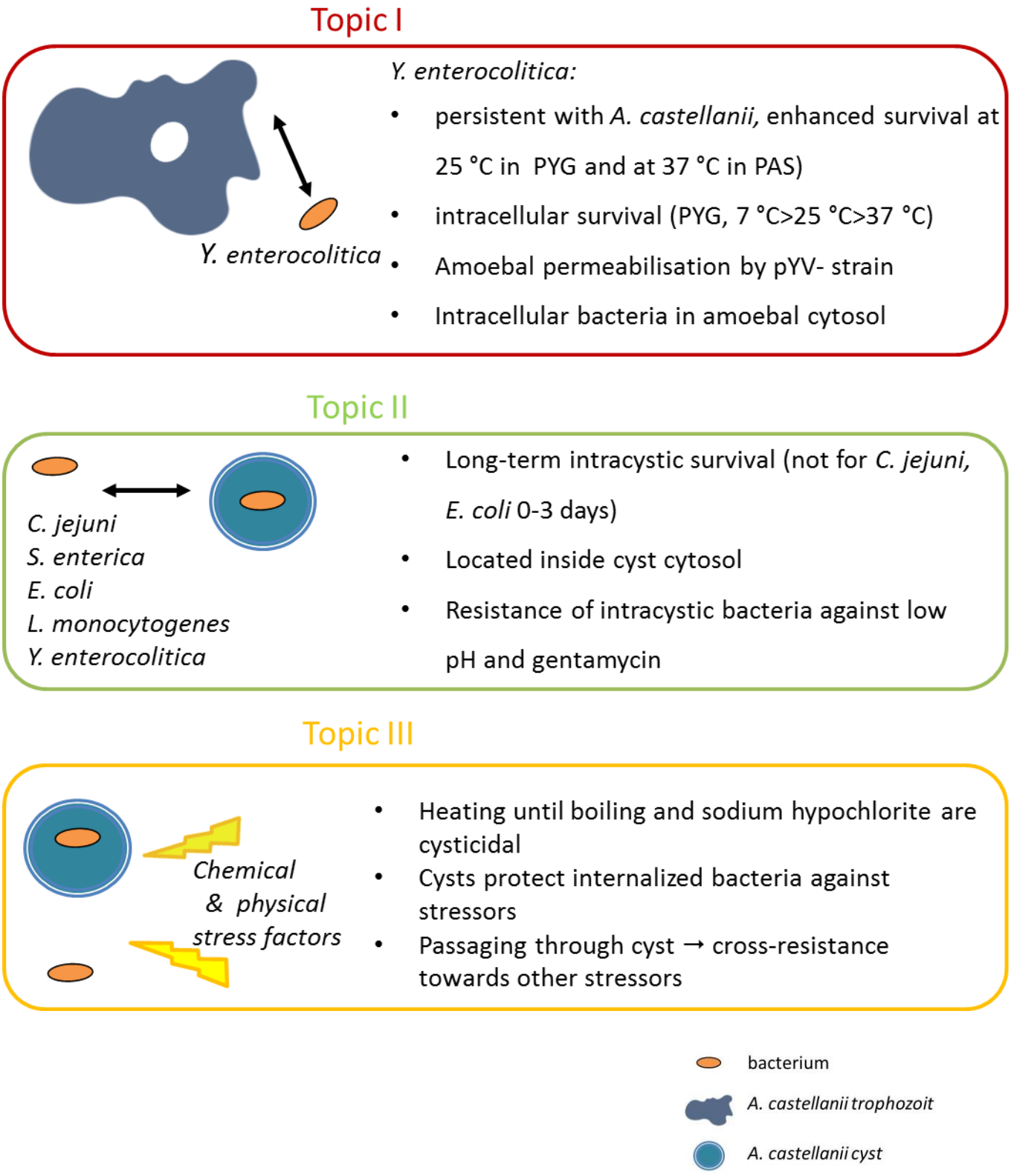

Figure 1: Overview of the results of this PhD thesis

At the start of this thesis, information regarding the impact and nature of the interaction between pathogenic *Y. enterocolitica* and FLA was limited compared to those of other foodborne pathogens (*C. jejuni*, *S. enterica*, *E. coli* and *L. monocytogenes*). Hence, we assessed the ability of *Y. enterocolitica* to grow/survive intra- or extracellularly with *Acanthamoeba* trophozoites under various environmental conditions (CHAPTER III). Once established that *Y. enterocolitica* - just like *C. jejuni*, *S. enterica*, *E. coli* and *L. monocytogenes* - could persist with amoebal trophozoites, we investigated the association of these five pathogens with amoebal cysts and the consequences thereof (CHAPTER IV-VI). First, various sample preparation protocols for TEM for detection of pathogenic foodborne bacteria inside cysts were developed and tested (CHAPTER IV). After determination of the bacterial uptake/invasion efficiency and long-term intracystic survival capacities, their intracystic location was determined with the optimized TEM protocol (CHAPTER V). As *S. enterica*, *Y. enterocolitica, E. coli and L. monocytogenes* could survive inside amoebal cysts, we further explored the sheltering capacity of cysts against various chemical and physical stressors frequently used in domestic and industrial food related environments (CHAPTER VI).

2. Challenges

This doctoral study only focusses on *Acanthamoeba* and hence only represent a tip of the bacteria-protozoa interaction-iceberg. Recent studies showed that food and food-related environments harbor an underestimated, but rich diversity of ciliates, amoebae and flagellates. Many of these organisms are not well described yet and experimental studies with environmental isolates are hampered by the current isolation techniques, which have a low recovery efficiency (Chavatte, 2016). In addition, finding the optimal axenic cultivation conditions to obtain sufficiently high cell numbers is often a long process of trial and error as there are no standardized protocols yet.

Our labs have extensive knowledge on the detection and identification of free-living protozoa in environmental samples, and have repeatedly tried to work with fresh environmental isolates. While we were often successful in culturing various protozoa, making an isolate amenable to controlled experimental manipulation is extremely difficult. In order to cultivate the environmental protozoa and reduce/eliminate bacterial and fungal contamination, dozens of subcultivation steps in artificial PYG-medium and the use of a mixture of antibiotics are required. For reasons of tractability and reproducibility, we worked with a culture collection *Acanthamoeba castellanii* strain (ATCC30234), although we are fully aware of the limitations of working with culture collections strains. Nevertheless, several studies proved that environmental amoebal strains are less sensitive towards biocides than laboratory strains that have been maintained in prolonged axenic culture (Turner et al., 2000; Hughes et al., 2003), justifying our hypothesis that the observed cyst resistance and their sheltering effect towards intracystic bacterial pathogens may even be an underestimation of what happens in real life. Our tests were performed in a laboratory environment without considering community aspects (e.g. background flora, selective predation), which for sure takes place under natural conditions on food and in food-related environments.

Moreover, the present studies included only a restricted set of foodborne pathogenic strains, and therefore formally may not reflect all possible forms of amoebae-bacteria interactions. The interactions we report in our study demonstrate concepts, but it remains to be shown whether they also takes place in natural settings.

Up to now, there is only one published report on the interaction between a foodborne pathogen and FLP in an *in situ* setting (Gourabathini et al., 2008). In this study, *S. enterica* and *Tetrahymena* were inoculated on cilantro leaves and observed under a confocal scanning microscope. *Salmonella enterica* was taken up, survived intracellular digestion and was subsequently expelled through vesicles. Some of the vesicles contained more than 20 bacteria and the authors suggested that at least some of these bacteria would be shielded from various physical and chemical stresses. Another study confirmed that *S. enterica* in expelled *Tetrahymena* vesicles are more resistant to chlorine than cells that remained free in suspension (Brandl et al., 2005).

For reasons of simplicity, traceability and reproducibility, most researchers perform *in vitro* cocultivation assays in controlled interaction settings. However even then conflicting results are observed. For instance, some researchers observe survival and/or replication of intra-amoebal bacteria, whilst others did not find intracellular viability of bacteria in protozoans. This can be explained by variations in (i) organisms (bacterial strains and protozoan strains, age, growth phase) (ii) coculture setup (temperature, multiplicity of infection, contact time between organisms, coculture medium) (iii) and analysis (recovery method, quantification, and viability determination of survivors) (Vaerewijck et al., 2014).

3. *Yersinia enterocolitica* persist with *Acanthamoeba castellanii*

The foodborne pathogen *Y. enterocolitica* and the free-living *Acanthamoeba* are encountered in the same habitats, a.o. environmental surface waters, meat processing environments (Fredriksson-Ahomaa et al., 2000; Vaerewijck et al., 2008; Bari et al., 2011; Mahmoudi et al., 2012) and the gastrointestinal system of pigs (Nesbakken et al., 2003; Chavatte et al., 2016a). Although there are no reports yet of yersiniae inside *Acanthamoeba in situ,* we consider that implying this ubiquitous amoebal model protozoon in cocultivation studies is highly relevant.

We demonstrated that several foodborne pathogenic yersiniae strains persist in association with the bacterivorous *A. castellanii* for at least 14 days *in vitro* and that this association enhanced bacterial survival. The latter observation was only detected under nutrient-rich cultivation conditions at room temperature and under nutrient-poor conditions at 37 °C. This confirms that fine-tuning of environmental parameters, such as temperature control and thorough cleaning procedures to reduce organic material, are relevant for pathogen control in food processing environments and slaughterhouses.
A recent study performed with *Yersinia pseudotuberculosis* demonstrated that these bacteria survive and replicate up to 1 week within *A. castellanii* trophozoites in nutrient rich PYG medium at room temperature and that this association resulted in prolonged survival compared to bacterial monocultures (Santos-Montanez et al., 2015). These findings confirm our earlier published results.

Transmission electron microscopy showed that intracellular *Y. enterocolitica* were located in the cytosol. This is in contrast with the recently published study of Santoz-Montanez where intra-amoebal *Y. pseudotuberculosis* were located inside amoebal vacuoles. The mechanisms by which *Yersinia* enter the amoebae, escape the digestion cycle and end up in the cytosol are not known yet. All our tested bacterial strains carried the *inv* (invasion) and *ail* (attachment invasion locus) genes, but only half of them carried the 70 kb-virulence plasmid (pYV). This plasmid contains genes encoding a Type three Secretion System (T3SS) and a variety of secreted protein effectors (Yops). The T3SS and it effectors are essential for *Legionella* to establish an intra-amoebal association, but apparently not for *Yersinia* as strains without the virulence plasmid were also able to reside inside the amoebae. Whereas

the *Yersinia* Yops are known to modulate macrophage signaling pathways, which leads to inhibition of phagocytosis and induction of apoptosis in macrophages, they seem to be dispensable for *Yersinia* uptake by/entering in *Acanthamoeba*. This may suggest that *Yersinia* employs different mechanisms to enter amoebae and macrophages.

4. Amoebal cysts: potential vector, shelter and training ground for foodborne pathogens

Cysts of free-living amoebae may be relevant for the ecology and epidemiology of pathogenic bacteria, given their tolerance for adverse environmental conditions and their high capacity for dispersal. It has been stated that FLP cysts are even more effective as a shelter and vector for internalized bacteria than trophozoites (Turner et al., 2000; Lloyd et al., 2001; Coulon et al., 2010; Dupuy et al., 2014).

In this thesis, for the first time, the intracystic survival of five major pathogenic bacteria was tested, along with the capacity of amoebal cysts to act as a vector, shelter and training ground (CHAPTERS V-VI). However, TEM protocols needed to be optimized first to be adequate for use towards amoebal cysts (CHAPTER IV), allowing a better visualization of the intracystic environment and locate intracellular bacteria.

4.1 TEM protocol for cysts fixation and visualization

Transmission electron microscopy is a valuable technique to visualize the intra-amoebal environment without major disturbance of intracellular structures. The paucity of information concerning TEM sample preparation for amoebal cysts makes detection and localization of intracystic bacteria a major challenge. Cysts of free-living protozoan often have a thick, robust, layered cell wall and are prone to stick strongly to the walls of plastic centrifugation tubes after fixation, resulting in failure to form a dense pellet. This leads to massive loss of cysts during the many centrifugation steps needed to prepare the TEM sample.

In collaboration with the group of Prof. W. Bert (Nematology research unit, UGent), who have ample expertise in TEM, we designed and tested four protocols for TEM sample preparation of *A. castellanii* cysts. Two protocols, one based on chemical fixation in collagen-coated well plates and one on HPF-AFS, were withheld as the most effective protocols for TEM-based ultrastructural studies of cysts. To our knowledge, this is the first time that High Pressure Freezing with Amplified Freeze Substitution (HPF-AFS) was used to visualize the intracystic environment. Though this protocol fixes the samples within

milliseconds and hence preserves also delicate fine structures, its major drawback is the long run-time (~ 1 week), making it difficult to analyze samples during short time interval studies. Both protocols resulted in high quality images of amoebal cysts. The proposed protocols will allow a better analysis of the cysts structures and a better understanding of bacterial survival mechanisms in cysts in future studies.

Collagen coating of well-plates was used to optimize cyst recovery during the TEM protocol, as collagen coating showed to favor adhesion of e.g. epithelial, endothelial and muscle cells, neurons (Nazarpour, 2013). As this procedure was also successful for amoebal cysts, we suggest that the TEM-protocols can also be used for protozoan cells, other than *Acanthamoeba,* and for weakly adherent cells or fragile cells (without the need for multiple centrifugation steps). Indeed, since its development, the chemical fixation protocol in the coated well plates has been successfully used by other researchers to prepare TEM samples of amoebal trophozoites, other than *Acanthamoeba*, which exhibited low adhesion capacities and were difficult to cultivate in high numbers (Van Wichelen et al., 2016).

In this doctoral thesis, the chemical fixation protocol with coated well plates, followed by TEM-analysis was further used in following experiments. TEM was used (i) to locate intracystic bacteria, (ii) to visualize both amoebal and bacterial structures during their interactions (Chapter IV), (iii) to evaluate the viability of intracystic bacteria by assessing the integrity of their internal structures and cell membranes. A major drawback of TEM in detecting intracystic bacteria is the requirement of fixed, embedded cross sections of the cells, which makes viability staining or time lapse experiments impossible. Another disadvantage, also related to the cross sections, is that exact counting of the total amount of internalized bacteria in a single amoeba is hard.

Working with fluorescent probes to detect and enumerate viable intracellular bacteria as an alternative for TEM is an idea we also considered, but it is difficult to accomplish as cyst walls are extremely robust, hampering probes to penetrate the cysts. Methods to open or permeabilize the cyst wall will effect the internal cyst environment and bacteria. Fluorescent labelling of bacteria by vector transformation before cocultivation could be an alternative. However, one has to examine if transformed bacteria have an altered interaction with amoebae compared to the native strain. Moreover, cysts are in a dormant stage, so it is

possible that intracellular bacteria are also in a metabolic resting stage, which might result in low expression of the vector during the intracystic stage of the bacteria. In addition, the cysts exhibit autofluorescence (Derda et al., 2009).

4.2 Intracystic survival of foodborne pathogens

Intracystic bacterial survival is highly relevant, as this may allow foodborne bacteria to survive the stringent cleaning and disinfection measures applied in food-related environments. In addition, those amoeba resistant bacteria (ARB) inside cysts are likely to be undetected by the standardized cultivation protocols for the detection of pathogenic bacteria in food-related environments.

The current study (CHAPTER V) proves that *Salmonella enterica, L. monocytogenes, Y. enterocolitica,* and *E. coli* can survive the encystment process and persist inside *Acanthamoeba* cysts for a long period of time. Intracystic bacteria survive even when exposed to antibiotic treatment (100 µg/ml gentamycin) or to highly acidic conditions (pH 0.2). Moreover, they can resume active growth in broth media following excystment. Whether these intracellular bacteria are released by a benign egress or by lysis of the amoebal host is not known yet.

While species-specific differences in trophozoite invasion/uptake efficiency were observed, these could not be correlated with the differences in intracystic survival period, ranging from 6 days after induction encystment for *E. coli O:157* up to 21 days for *S. enterica*. Cultivable *C. jejuni* could never be retrieved after induction of excystment. Demonstrating the presence of viable (cultivable) bacteria inside *A. castellanii* cysts by plating the intracystic suspension on agar plates was not possible due to resistance of amoebal cysts to lysis (El-Etr et al., 2009). Lysis of cysts can be accomplished by a combination of autoclaving, beat beating and proteinase K, but these techniques of course also kill the intracellular bacteria.

Hitherto, quantitative and qualitative data about protozoan cysts on food or in food-related environments is still scarce. Unlike foodborne pathogens, free-living protozoa are not monitored in food processing plants as they are regarded as harmless. However, several studies report the presence of an extensive variety of free-living protozoa in these habitats. For instance, at least 61 species could be detected in meat cutting plants and over 100 species in domestic refrigerators (Vaerewijck et al., 2008; Vaerewijck et al., 2010). Unfortunately, no quantification of FLP was performed in these studies. Moreover, these studies were based on 18S rDNA sequencing analysis or on enrichment by cultivation and subsequent identification, hence no distinction could be made between cysts and

trophozoites. Food-processing plants and other food-related environments are often regarded as hostile environments for free-living protozoa due to frequent cleaning and disinfection, desiccation and low processing/storage temperatures. However, these factors might actually select colonization by cyst-forming protozoa.

Cysts harbor and protect internalized pathogens, this raises the question if cysts as such pose a direct threat to public health. Up to 53% of the *A. castellanii* cysts were infected with pathogenic bacteria, which were located in the cyst cytosol (CHAPTER V). The mean number of intact bacteria inside a cyst cross section varied between 1.4 and 2.6. As the estimated infective dose (*i.e.* the number of bacteria required to successfully infect a host) ranges from 10 bacteria for EHEC up to 10^6 for *Y. enterocolitica*, bacterial infection can only occur (i) when multiple cysts are ingested and excystment takes place in the gastrointestinal system or (ii) when bacteria from a single ingested cyst are able to grow upon release after excystment. Currently, no information is available whether cysts are able to excyst in the human gastrointestinal system. *In vitro* excystment in PYG-medium takes 2 days, which might be too slow to release intracellular bacteria in the gastrointestinal tract, given the speed of the human digestion system. However, studies performed on cysts of the protozoan parasite *Giardia* demonstrate that a low (stomach) pH and intestinal proteases may stimulate excystment (Boucher et al., 1990). While no data are available about the numbers of protozoan cysts on food and in food-related environments, it has been shown that amoebae in general can reach up to 9.3 10^3 MPN/g lettuce (Vaerewijck et al., 2011), and up to 10^4MPN/g sprouts (Chavatte et al., 2016b). The majority of the detected species had the capacity to form cysts. Little is known about the influence of environmental factors on en- and excystment in foodrelated habitats. Lakhundi (2014) demonstrated that excystment of *Acanthamoeba* was optimal at neutral pH at 30°C and was reduced at pH4 and 9 and at 4°C On the other hand, addition of 5% CO_2 stimulated excystment.

4.3 *Acanthamoeba* cysts enhance tolerance of foodborne pathogenic bacteria to stressors of food related environments.

A recurrent question in food safety is how foodborne pathogens survive the harsh sanitation procedures in food processing plants. Localization in hidden microniches on food or on processing equipment, and formation of biofilms, has been suggested as possible stress avoidance mechanisms (Brandl et al., 2005). The role of protozoan communities in the persistence of bacterial foodborne pathogens remains however largely unexplored.

In the last part of this doctoral thesis, we observed an increased bacterial resistance towards various chemical and physical stressors when the foodborne pathogenic bacteria were located inside *Acanthamoeba* cysts. This protective effect is of increasing concern as it is speculated that biocide treatments might actually select for tolerant FLA and hence also their intracellular microorganisms. In addition, we detected that exposure to the intracystic environment enhances bacterial resistance to other stress factors after excystment. This suggests similar stress response mechanisms to the intracystic environment and external stressors. Future experiments need to reveal how long this acquired cross-resistance lasts, which is the range of stressors this cross-resistance may apply to, and what are the mechanisms behind this cross-resistance

Despite increasing concerns about their role as hosts for other microorganisms, there are currently no standard methods for the inactivation of free-living amoebae. Cysts can be a principal target for disinfection programs. Killing the cysts or avoiding the formation of cysts may hamper potential bacterial protection and transmission. Results of this PhD study indicate that hypochlorite treatment and heating until boiling is cysticidal. Potential targets for inhibition of encystment are e.g. enzymes that are crucial during encystment, such as xylose isomerase and cellulose synthetase (Aqeel et al., 2013; Moon et al., 2014).

Antimicrobial testing against free-living amoebae is compounded by the absence of standardized methods (Thomas et al., 2010). The efficacy of a limited number of biocides against *Acanthamoeba* have been investigated in studies mainly focusing on medical applications (contact lenses) or water disinfection (Thomas et al., 2010). Treatments such as isopropyl alcohol (20%, 6h), propylhexamethylene biguanide (0.02%, 24h) formalin (10%, 30min, (Aksozek et al., 2002)), and peracetic acid (150 mg/l, 18h, (Greub and Raoult, 2003)),

have been shown to be cysticidal to some extent. Some discrepancies in results have been observed. This can be due to strain differences, altered encystment conditions or the applied test protocol. Moreover these studies do not take into account organic matter such as oils, fat and protein from food residues. These factors can prevent sanitizers from coming into physical contact with the surface to be sanitized, and the target organisms (being bacterial pathogens or amoebal hosts or their interaction-stage). In addition, the presence of organic matter inactivates or reduces the effectiveness of some types of sanitizers, making sanitization ineffective (Vaerewijck et al., 2012).

5. Future perspectives

The results obtained during this PhD research underline the need to further advance our knowledge on prokaryote and eukaryote microbial diversity and interactions on food and in food-related environment in order to improve control measures for foodborne pathogens. Hitherto, a comparative study between the major food and food-related environments sampled revealed the presence of a core protozoan community (Chavatte, 2016 and references herein). This core community is composed of several amoebae including *Acanthamoeba* spp., vannellids, hartmannellids, vahlkampfiids and *Vermamoeba vermiformis*. Their ecological significance and function in the persistence of pathogens in food-related environments remains unknown. Free-living protozoa could influence the habitat diversity by selective grazing and by promoting persistence of certain bacteria. In the future, optimized, standardized isolation and cultivation protocols for free-living protozoa need to be developed. These will be complementary with molecular analyses e.g. metagenomics and metatranscriptomics in revealing prokaryotic and eukaryotic diversity and setting up interaction networks. This will enable us to examine (i) how ecological interactions affect pathogen occurrence and dynamics in food-processing environments and (ii) if FLA can act as a reservoir, vector, shelter and training ground *in situ*.

The present research demonstrated that several foodborne bacterial pathogens resist digestion by *Acanthamoeba castellanii* trophozoites and survive inside the sheltering cysts under harsh conditions. Moreover, bacterial passaging through amoebal cysts can induce cross-tolerance towards other stresses such as ethanol, hypochlorite, hydrogen peroxide and NaCl treatments. To further assess the impact of cysts on human health and food safety, future research is necessary to detect and quantify cysts of FLP (amoebae, ciliates and flagellates) in food-related environments and to reveal if environmental factors might trigger excystment *in situ*. Moreover, factors and conditions which may prevent uptake of foodborne pathogens by free-living protozoa and subsequent protozoan encystation need to be identified in order to decrease bacterial survival and persistence. To be able to compare and extrapolate results, standardized interaction protocols need to be designed.

Up till now, the majority of the interaction studies are performed with one bacterial and one amoebal species, but in real ecosystems multispecies interactions occur, indicating the need

for future microbiome studies. In addition, most of published studies are performed in suspension, but bacteria can also occur as adherent cells and are often organized in biofilms. Protozoan feeding preferences and their impact on (food-related) biofilms is not well understood. It has been shown that protozoan grazing is a major factor in controlling fresh water biofilm community composition (Dopheide et al., 2011). Own preliminary research confirmed that FLP can influence initiation and formation of biofilms of food related bacterial strains.

In conclusion, this PhD research contributes to the observations that interactions between free-living protozoa and microorganisms can critically influence the persistence of pathogens. This illustrates the need to broaden our view on single species experiments and to unravel the complexity of interspecies interactions.

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SUMMARY

Despite thorough disinfection protocols and hygiene monitoring during food production and processing, human pathogenic bacteria often persist in food related environments and on food. The **foodborne pathogenic bacteria** *Campylobacter jejuni*, *Salmonella enterica*, verocytotoxigenic *Escherichia coli, Yersinia enterocolitica*, and *Listeria monocytogenes* are among the most frequently reported causes of foodborne illness. These foodborne bacterial infections cause gastrointestinal illnesses, characterized by fever, diarrhea, abdominal cramps, nausea, and vomiting, but sometimes even (lethal) complications can occur. In order to prevent these diseases, it is important to identify the possible reservoirs and transmission routes of these pathogens.

In recent years, it has become increasingly clear that **free-living amoebae** may act as a reservoir, vector, protective host and virulence training ground for bacteria. Free-living amoebae such as *Acanthamoeba castellanii* are unicellular, eukaryotic microorganisms which share the same environmental and anthropogenic niches as foodborne pathogens. Hence, there is a growing concern that they may play an as yet underestimated role in the contamination, transmission and persistence of pathogenic bacteria in food related environments.

The general aim of this thesis was to gain more insight in the interactions between the free-living, ubiquitous model amoeba, *A. castellanii* and the foodborne bacterial pathogens *C. jejuni*, *S. enterica*, *Y. enterocolitica*, *E. coli* and *L. monocytogenes.*

In CHAPTER I, a comprehensive overview is provided on the characteristics, classification and ecology of free-living amoebae. Special attention is given to their interaction with grazing resistant bacteria and the associated benefits for both amoebae or bacteria. Furthermore, a concise review of foodborne bacterial pathogens is presented and their known interactions with free-living amoebae are discussed. Finally the conceptual framework and research aims of this thesis are formulated (CHAPTER II).

CHAPTER III exclusively focuses on the interaction between *Acanthamoeba* trophozoites and *Y. enterocolitica* under various environmental conditions, since information about this specific interaction was scarce. Results indicate that the four tested *Y. enterocolitica* strains resisted predation by *A. castellanii* for at least 14 days, irrespective of medium (nutrient rich/poor) and temperature (7, 25 and 37°C). Association with *A. castellanii* significantly

enhanced the survival of the *Yersinia* strains under nutrient rich conditions at 25°C (+ 2.4 Log_{10} after 14d) and under nutrient poor conditions at 37°C (+ 1 Log_{10} after 14d). Furthermore, factors excreted by one *Y. enterocolitica* strain showed a temperature-dependent permeabilizing effect on the amoebae.
Long-term intraprotozoan survival of *Y. enterocolitica* depended on nutrient availability and temperature, with up to 2.8 log cfu/ml bacteria surviving intracellularly at 7°C for at least four days in nutrient rich medium. Transmission electron microscopy revealed that intra-amoebal yersiniae were located in the amoebal cytosol.

The production of resistant, dormant cysts forms an integral part of the life cycle of many free-living protozoa, allowing these organisms to survive adverse environmental conditions. In the second part of this thesis, the ability of foodborne bacterial pathogens to survive inside amoebal cysts and resist stressful conditions is investigated.

CHAPTER IV deals with the detection and localization of intracystic bacteria. While Transmission Electron Microscopy (TEM) is ideally suited for these analyses, conventional TEM protocols tend to result in low cyst yield and images of poor quality, making them not optimal for further cell biological analysis. In this study, four different protocols for TEM sample preparation of cysts were designed and tested. Two protocols, one based on chemical fixation in collagen coated well plates and one on High Pressure Freezing with Automatic Freeze Substitution, were selected as the most effective for TEM-based ultrastructural studies of cysts. These protocols will allow a better analysis of the cyst structures and a better understanding of bacterial survival mechanisms in cysts.
In CHAPTER V we show that *S. enterica, Y. enterocolitica, E. coli* and *L. monocytogenes* can survive inside amoebal cysts and resume active growth after excystment, even when they have been exposed to antibiotic treatment and highly acidic conditions. Strain- and species-specific differences in survival period were observed, with *S. enterica* surviving up to three weeks inside the amoebal cysts. These differences were not related to variation in trophozoit invasion/uptake efficiency. The viability and the encystation process of the amoebae was not markedly affected by bacteria, except for strain *Y. enterocolitica* 2/O:9. For this strain a significant delay in encystment was observed.

Transmission electron microscopy revealed that up to 53% of the cysts were infected with pathogenic bacteria, which were located in the cyst cytosol. Apparently intact cells of another common bacterial pathogen, *C. jejuni,* were observed inside *A. castellanii* cysts, but no viable bacteria were observed after excystment.

CHAPTER VI further explores the sheltering function of amoebal cysts. Several physical and chemical stressors frequently used in domestic and industrial food related environments were tested, but only heating until boiling and sodium hypochlorite treatment were cysticidal. Other stressors were ineffective in reducing cyst viability and bacteria residing inside cyst were also more tolerant towards these stressors compared to bacterial monocultures. Even after release into the environment, increased tolerance towards subsequent H_2O_2 and 70% ethanol treatment was observed for all tested bacteria. Moreover, intracystic passage significantly increased the survival of *Y. enterocolitica, E. coli* and *S. enterica* after NaCl treatment and of *E. coli*, *S. enterica* and *L. monocytogenes* after sodium hypochlorite treatment compared to non-primed bacteria. This indicates that survival inside cysts may induce cross-tolerance in bacteria.

In conclusion, this PhD thesis demonstrates that the foodborne pathogens *S. enterica*, *Y. enterocolitica, E. coli* and *L. monocytogenes* survive inside *A. castellanii* trophozoites/cysts. Cysts can act as a vector and shelter for internalized bacteria. Moreover, survival inside cysts can induce cross-tolerance to subsequent exposure of various chemical and physical stressors.

These findings identify a potential role of free-living amoebae cysts in the ecology and epidemiology of pathogenic foodborne bacteria.

SAMENVATTING

Ondanks grondige desinfectieprotocols en hygiëne monitoring tijdens voedselproductie en -verwerking blijven humaan pathogene bacteriën aanwezig in voedselgerelateerde omgevingen en op voedsel. De **bacteriële voedselpathogenen** *Campylobacter jejuni*, *Salmonella enterica*, verocytotoxigene *Escherichia coli*, *Yersinia enterocolitica* en *Listeria monocytogenes* behoren tot de meest frequent gerapporteerde oorzaken van voedsel overgedragen infecties. Deze voedselgerelateerde bacteriële infecties veroorzaken gastro-intestinale klachten zoals koorts, diarree, buikkrampen, misselijkheid en braken. Soms kunnen lethale complicaties optreden. Om deze infecties te vermijden is het belangrijk om de mogelijke reservoirs en overdrachtsroutes van deze pathogenen te achterhalen.

De laatste jaren wordt het steeds duidelijker dat **vrijlevende amoeben** een reservoir, vector, beschermde gastheer en virulentie trainingskamp voor bacteriën kunnen zijn. Vrijlevende amoeben zoals *A. castellanii* zijn ééncellige, eukaryote micro-organismen die voorkomen in dezelfde natuurlijke en antropogene niches als voedselpathogenen. Daardoor is er een toenemende bezorgdheid dat hun rol in de besmetting, overdracht en persistentie van pathogene bacteriën in voedselgerelateerde omgevingen onderschat wordt.

Deze thesis had als algemeen doel meer inzicht te verwerven in de interacties tussen de vrijlevende, alomtegenwoordige modelamoebe, *A. castellanii*, en de bacteriële voedselpathogenen *C. jejuni*, *S. enterica*, *Y. enterocolitica*, *E. coli* en *L. monocytogenes*.

In HOOFDSTUK I wordt een uitgebreid overzicht gegeven van de eigenschappen, classificatie en ecologie van vrijlevende amoeben, met speciale aandacht voor hun interactie met graas-resistente bacteriën en de daarmee geassocieerde voordelen voor amoeben en bacteriën. Verder wordt er een beknopt overzicht gegeven van voedselpathogenen en worden de interacties met vrijlevende amoeben bediscussieerd. Tenslotte wordt het conceptueel kader en de onderzoeksdoelstellingen van deze thesis geformuleerd (HOOFDSTUK II).

HOOFDSTUK III focust uitsluitend op de interactie tussen *Acanthamoeba* trophozoieten en *Y. enterocolitica* onder verschillende omgevingscondities, aangezien informatie over deze specifieke interactie schaars was. Uit de resultaten blijkt dat de vier geteste *Y. enterocolitica* stammen resistent waren tegen predatie van *A. castellanii* gedurende minstens 14 dagen, ongeacht het medium (nutriëntarm/-rijk) en de temperatuur (7, 25 en 37°C). Associatie met *A. castellanii* verhoogde de overleving van de *Yersinia* stammen in nutriëntrijke condities bij

25 °C (+ 2.4 Log_{10} na 14d) en in nutriëntarme condities bij 37 °C (+ 1 Log_{10} na 14d) significant. Bovendien bleek dat factoren gesecreteerd door één *Y. enterocolitica* stam een temperatuursafhankelijk permeabilizerend effect hadden op de amoeben.
Langdurige intraprotozoaire overleving van *Y. enterocolitica* was afhankelijk van nutriënt beschikbaarheid en temperatuur, waarbij tot 2.8 log cfu/ml bacteriën voor minstens vier dagen intracellulair konden overleven bij 7°C in nutriënt rijk medium. Transmissie electronen microscopie toonde aan dat intracellulaire yersiniae gelokaliseerd zijn in het cytosol van de amoeben.

De productie van resistente, dormante cysten maakt integraal deel uit van de levenscyclus van vele vrijlevende protozoa. Dit laat hen toe om in ongunstige omstandigheden te overleven. In het tweede deel van deze thesis wordt onderzocht of bacteriële voedselpathogenen in amoebe cysten kunnen overleven en of ze kunnen weerstaan aan stressvolle condities.

HOOFDSTUK IV handelt over de detectie en lokalisatie van bacteriën in cysten. Hoewel transmissie elektronen microscopie (TEM) ideaal is voor deze analyses, zorgden de conventionele TEM-protocols voor een lage cysten opbrengst en beelden van slechte kwaliteit die niet geschikt waren voor verder celbiologische analyses. In deze studie werden vier verschillende TEM staalbereiding protocols voor cysten ontworpen en getest. Twee protocols, één gebaseerd op chemische fixatie in collageen gecoate wellplaten en één op High Pressure Freezing met Automatic Freeze Substitution werden geselecteerd als zijnde het meest effectief voor TEM gebaseeerde ultrastructurele studies van cysten. Deze protocols zullen een betere analyse van cyststructuren toelaten en leiden tot een betere kennis van bacteriële overlevingsmechanismen.

In HOOFDSTUK V wordt aangetoond dat *S. enterica*, *Y. enterocolitica*, *E. coli* en *L. monocytogenes* in cysten van amoeben kunnen overleven. Na excystering groeien deze bacteriën terug, zelfs wanneer ze blootgesteld werden aan antibiotica en sterk zure condities. Stam en species-specifieke verschillen in overlevingsperiode werden geobserveerd, waarbij *S. enterica* tot drie weken kon overleven in de cysten. Deze verschillen waren niet gerelateerd aan de variatie in de trophozoit invasie/opname efficiëntie. De viabiliteit van de amoeben en het encysteringsproces werd niet beïnvloed

door de aanwezigheid van bacteriën, behalve bij *Y. enterocolitica* stam 2/O:9. Bij deze stam werd er een significante vertraging in encystering geobserveerd.
Transmissie electronen microscopie toonde aan dat tot 53% van de cysten geïnfecteerd waren met pathogene bacteriën. Deze bacteriën waren gelokaliseerd in het cyst cytosol. Schijnbaar intacte cellen van *C. jejuni*, een andere bacteriële pathogeen, werden geobserveerd in *A. castellanii* cysten, maar er werden geen leefbare bacteriën geobserveerd na excystering.

Hoofdstuk VI onderzoekt verder de beschermende functie van amoebe cysten. Verscheidene fysische en chemische stressfactoren die frequent gebruikt worden in huishoudelijke en industriële voedselgerelateerde omgevingen werden getest, maar enkel verwarmen tot het kookpunt en sodium hypochloriet behandeling waren cysticidaal. Andere stressfactoren waren niet effectief in het reduceren van de levensvatbaarheid van de cysten. Bacteriën in de cysten waren ook meer tolerant voor deze stressfactoren in vergelijking met bacteriële monoculturen. Zelfs na vrijgave in de omgeving werd een toegenomen tolerantie voor H_2O_2 en 70% ethanol geobserveerd voor de geteste bacteriën. Bovendien leidde passage doorheen cysten tot een significant verhoogde overleving van *Y. enterocolitica, E. coli* en *S. enterica* na NaCl behandeling en van *E. coli*, *S. enterica* en *L. monocytogenes* na sodium hypochloriet behandeling in vergelijking met niet geprimede bacteriën. Dit duidt erop dat overleving in cysten kan leiden tot kruistolerantie in bacteriën.

Uit deze thesis kan geconcludeerd worden dat de voedselpathogenen *S. enterica, Y. enterocolitica, E. coli* en *L. monocytogenes* overleven in *A. castellanii* trophozoiten/cysten. Cysten kunnen dienst doen als vector en beschermende gastheer voor geïnternaliseerde bacteriën. Bovendien kan overleving in cysten kruistolerantie induceren voor een volgende blootstelling aan chemische en fysische stressfactoren.
Deze bevindingen tonen aan dat vrijlevende protozoa een tot hiertoe weinig gekende rol kunnen hebben in de ecologie en epidemiologie van bacteriële voedselpathogenen.

Curriculum vitae

Ellen Lambrecht werd geboren op 12 mei 1989 te Deinze. Na het beëindigen van haar secundaire studies Wetenschappen-Wiskunde aan het O-L-V-Hemelvaartinstituut te Waregem, startte ze in 2007 met de studies Biochemie en Biotechnologie aan de Universiteit Gent. Zij behaalde in 2012 het diploma van Master in de Biochemie en Biotechnologie (major Microbiologie) met grootste onderscheiding en kreeg de Devgen award voor meest bewonderenswaardige carrière in de Biochemie en Biotechnologie. In september 2012 startte zij als bursaal van het Bijzonder Onderzoeksfonds van de UGent haar doctoraatsonderzoek bij de vakgroep Veterinaire Volksgezondheid en Voedselveiligheid van de faculteit Diergeneeskunde. Dit onderzoek handelde over de interacties tussen vrijlevende amoeben en bacteriële voedselpathogenen. Het onderzoek werd begeleid door Prof. Dr. K. Houf, Prof. Dr. K. Sabbe en Dr. J. Baré. In 2014 won zij de BSFM prijs voor 'excellence in food microbiology research' en tevens vervolledigde zij in 2016 het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de UGent. Ellen Lambrecht is auteur en mede-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Zij nam actief deel aan meerdere nationale en internationale congressen.

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